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# AN EVALUATION OF IN VITRO TESTS IN THE DIAGNOSIS OF VIRUS DISEASES

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THE neutralization test is the laboratory procedure now in general use for the diagnosis of virus diseases. Since this test is cumbersome and costly, as well as time-consuming, efforts have been made in recent years to replace it with methods which would be simple, accurate, and inexpensive. The complement fixation test recently described by Casals and Palacios<sup>1</sup> and by Havens and his associates<sup>2</sup> is sufficiently specific to differentiate the various experimental infections caused by the neurotropic viruses. This test has already yielded encouraging results<sup>3, 4</sup> when used in the diagnosis of human infections induced by some of these viruses. Meanwhile, certain other in vitro antigen-antibody tests have been described by Goodner,<sup>5</sup> Cannon and Marshall,<sup>6</sup> and Roberts and Jones,<sup>7-9</sup> but no attempts to determine the value of these tests as diagnostic aids seem to have been published to date. It appeared that comparison of the efficiency of these tests with that of the aforementioned complement fixation and neutralization tests might lead to further simplification of laboratory methods. In the present study, therefore, attempts have been made to ascertain the relative value and practicability of these procedures in the diagnosis of virus diseases. In a few experiments with the influenza virus, the Hirst<sup>10</sup> red cell agglutination-inhibition test was also included in this comparison.

## MATERIALS AND METHODS

All antigens were freshly prepared and the technique as outlined by the authors of the respective tests was rigidly adhered to in all details. Antigens used in this study were prepared with the viruses of eastern and western equine encephalomyelitis,<sup>1, 5-7</sup> St. Louis encephalitis,<sup>1, 5-7</sup> rabies,<sup>1, 5-7</sup> lymphogranuloma venereum,<sup>5-7, 11</sup> and type A influenza.<sup>5-7, 12</sup> Human as well as hyperimmune animal sera† were tested. In a few instances hyperimmune mouse serum was obtained by injecting animals with homologous brain tissue and pooling the samples of blood. In the complement fixation tests with the neurotropic viruses, all human, goat, and mouse sera were heated to 60° C. for twenty minutes before using. Guinea pig and rabbit sera, respectively, were inactivated for twenty minutes at 56° C. In the complement fixation tests with the viruses of epidemic influenza and lymphogranuloma venereum the sera were inactivated for thirty minutes at 56° C.

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TABLE I

RESULTS OF AGGLUTINATION, COMPLEMENT FIXATION, AND NEUTRALIZATION TESTS ON REPRESENTATIVE SERUMS

ANTIGENS		SERA*	TEST				
VIRUS	INFECTED TISSUE		COLLIDION-FIXATION (GOODNER <sup>3</sup> )	COLLIDION- AGGLUTINATION (CANNON-MARSHALL <sup>6</sup> )	B. A. METHOD (ROBERTS-JONES <sup>7</sup> )	COMPLEMENT FIXATION <sup>1, 11, 12</sup>	NEUTRALIZATION <sup>13-15</sup>
St. Louis encephalitis (Taylor strain)	Mouse brain	Rabbit	⊕	+	+	ac	+
		Horse†	⊕	⊕	⊕	ac	+
		Mouse	⊕	⊕	⊕	+	+
		Human†	⊕	+	⊕	⊕	⊕
Western equine encephalomyelitis (California strain)	Mouse brain	Guinea pig	○	○	○	+	+
		Rabbit	ns	ns	ns	+	+
		Horse†	⊕	+	+	+	+
		Human†	ns	ns	ns	ac	+
		Human†	⊕	⊕	⊕	+	+
		Human§	⊕	○	⊕	○	⊕
Eastern equine encephalomyelitis (TenBroeck strain)	Mouse brain	Guinea pig	⊕	+	+	+	+
		Rabbit	○	○	○	○	⊕
		Rabbit	⊕	⊕	⊕	ac	+
		Guinea pig	⊕	+	⊕	⊕	⊕
Rabies (No. 5557)	Mouse brain	Guinea pig	⊕	+	⊕	+	⊕
		Rabbit	+	⊕	+	ac	+
		Goat	⊕	+	⊕	⊕	⊕
		Human	⊕	+	+	+	+
		Human†§	○	⊕	○	○	○
		Human†	+	+	⊕	+	+
Influenza (PR 8)	Mouse lung	Human†	+	⊕	⊕	+	+
		Human†	⊕	+	⊕	+	+
		Human**	⊕	+	⊕	○	+
		Human**	+	⊕	⊕	+	+
	Allantoic fluid	Ferret	+	+	+	+	+
		Human†	+	⊕	+	+	+
Lymphogranuloma venereum (J)	Yolk sac	Goat	ns	ns	ns	ac	nt
		Human†	+	+	⊕	⊕	nt
		Human†	+	+	+	+	nt
		Human†	+	+	⊕	⊕	nt

\*Unless otherwise stated, the sera were obtained by hyperimmunizing animals.

†Natural infection; convalescent phase serum sample.

‡Tested previously by Hammon and Izumi and found to contain neutralizing antibodies against the virus of St. Louis encephalitis.

§Person vaccinated against western equine virus.

||Serum was obtained twenty days after exposure and eleven days after completion of Pasteur treatment.

||Patient with clinical rabies; no Pasteur treatment; serum obtained thirty-nine days after exposure.

\*\*Vaccinated with influenza a complex vaccine.<sup>16</sup>

+ Indicates a positive test.

⊕ Indicates a doubtful test; weakly positive neutralization test.

○ Indicates a negative reaction.

ac, Serum anticomplementary.

ns, Nonspecific reaction; namely, reaction with test antigen and with control.

nt, Not tested.

The neutralization tests with the viruses of St. Louis encephalitis and equine encephalomyelitis were performed according to the method previously described by Hammon and Izumi,<sup>13</sup> while with the rabies virus the procedure of Webster<sup>14</sup> was used. The technique of the neutralization test with the influenza

virus was essentially the same as that described by Lennette and Horsfall.<sup>15</sup> No neutralization tests were conducted with the agent of lymphogranuloma venereum. In reading the agglutination tests, the method of resuspension-agglutination was used since this procedure tends to eliminate prozone effects. The suspension of collodion particles, together with the antigen-antibody system in question, is centrifuged, thus bringing the particles together.<sup>6</sup>

### RESULTS

Some of the tests herein reported were conducted during the latter part of 1941 and involved the use of sera which had been on hand since 1937. The results of the tests on representative serum specimens from various natural and induced virus infections and from immunized animals are summarized in Table I. Few of the sera tested gave positive results with all of the tests used. In the majority of instances there was agreement between the results of the complement fixation and neutralization tests, while in only a few instances did the results of agglutination tests agree with those obtained by the other procedures. The serum from one of the workers (E. M. I.) who had been previously vaccinated against the western equine virus contained neutralizing antibodies, but no complement-fixing or agglutinating antibodies could be demonstrated. A number of sera from individuals inoculated<sup>16</sup> with the complex influenza a-dissemper chick embryo vaccine of Horsfall and Lennette<sup>17</sup> were also tested by the various methods, using infected mouse lung tissue in preparing the antigens. The results with only two of these sera are presented in Table I. Although no complement-fixing antibodies could be demonstrated in one of these serum samples, both neutralizing and agglutinating antibodies were detectable. Aside from the serum which failed to show complement-fixing antibodies, a remarkable degree of correlation was noted with influenza virus and the corresponding serum. Incidentally, all of these gave positive reactions with the Hirst red cell agglutination-inhibition technique.<sup>10</sup>

With the rabies and influenza viruses suggestive flocculation occurred with some sera even without the use of collodion particles or bacterial cells. These observations are in agreement with those previously reported by Havens and Mayfield<sup>18, 19</sup> and by Magill and Francis.<sup>20</sup> Henle and Chambers<sup>21</sup> also observed flocculation when the pooled extraembryonic fluids of developing chick embryos inoculated with influenza a virus were mixed with human or ferret convalescent sera.

The frequent lack of agreement between the complement fixation and neutralization tests has been demonstrated previously<sup>4</sup> and may be influenced by the time during convalescence when the blood specimens are obtained. The temperature at which the serum is stored may likewise influence the results.<sup>2</sup> Neutralizing antibodies against the western equine encephalomyelitis virus usually may be detected earlier than the complement-fixing antibodies, while in St. Louis encephalitis the reverse is true.<sup>4, 22, 23</sup> Furthermore, the complement-fixing antibodies persist longer in equine encephalomyelitis than they do in St. Louis encephalitis.<sup>23</sup>

Many factors appear to complicate the procedure involving the use of collodion particles or bacterial cells. Apparently particles 24 hours old or older

often flocculate spontaneously in the presence of serum proteins (especially whole horse serum); hence low serum dilutions may yield false positive results. Also, fresh human serum contains factors which may cause spontaneous flocculation. This, however, may be eliminated by inactivating the serum at 56° C. for thirty minutes. Similar difficulties present themselves also when bacteria instead of collodion particles are used (Roberts-Jones test). Spontaneous clumping frequently occurs in the freshly prepared suspensions of bacterial cells killed by flowing steam. This was demonstrated by examination of stained preparations.

#### SUMMARY AND CONCLUSIONS

An analysis of these findings indicates that the Goodner collodion-fixation test, the Cannon and Marshall collodion-agglutination test, and the Roberts and Jones bacterial agglutination method, though simple in operation, present too many complicating factors to permit of general use as routine procedures. Because of the ease with which many sera can be examined and because of the relative simplicity of the technique, the complement fixation test,\* whether used alone or in conjunction with the neutralization test, is eminently useful as a method for diagnosis of virus diseases.

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\*At the present time antigens are being prepared and tested for their usefulness with the view of recommending their preparation on a commercial scale for distribution to laboratories.

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## THE CEPHALIN-CHOLESTEROL FLOCCULATION TEST

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AN INTEREST in the treatment of cirrhosis of the liver has led quite naturally to a consideration of the value of liver function tests as an aid to the early diagnosis of cirrhosis and as a measure of change in hepatic function produced by therapy. The known limitations of the older tests prompted us to investigate the suitability of the cephalin-cholesterol flocculation test.

The cephalin-cholesterol flocculation test was introduced in 1938 by Hanger.<sup>1</sup> He described the preparation of the cephalin-cholesterol emulsion as well as the results obtained in a variety of diseases. No significant flocculation was observed in the absence of liver disease, and variable degrees of flocculation (depending upon the severity of the lesion) were consistently found in the presence of hepatic disease. Hanger and others,<sup>2, 4-12</sup> have confirmed these findings and have established beyond any doubt the usefulness of the test. Differences of opinion have arisen, however, concerning the value of the test in the presence of other systemic disease, its correlation with other liver function tests, its usefulness in differentiating obstructive from nonobstructive jaundice, the mechanism of a positive reaction, and other problems. The possibility that the present data may contribute to a better understanding of some of these problems would seem to justify this communication.

### MATERIAL

From among the 1,500 tests reviewed, 500 were chosen for this analysis. They were done on the sera of patients whose diagnoses have been established in most instances at necropsy or at operation. The remaining diagnoses are supported by unmistakable clinical evidence. The cases have been classified in three groups:

- I. Patients with known lesions of the liver
  - a. Diffuse
  - b. Discrete
- II. Patients with suspected or probable dysfunction of the liver
- III. Patients with no known lesion or dysfunction of the liver

### METHOD

Inasmuch as not more than from ten to fifteen tests were done per week, it was found advantageous to do them only one morning each week. This simplified the laboratory work and prevented needless waste of reagents.

<sup>1</sup>Presented before the Seventeenth Annual Meeting of the Central Society for Clinical Research, Chicago, Nov. 4, 1944.

From the Department of Medicine and the Snodgrass Laboratories of the St. Louis City Hospital, and the Department of Medicine, Washington University School of Medicine. Received for publication, Oct. 2, 1944.

The procedure was as follows:

A stock ether solution was prepared by placing 8 c.c. of ether in the vial containing the cephalin-cholesterol mixture\* and shaking thoroughly. The resultant stock ether solution was stored in the refrigerator for use as needed.

Not more than a few hours prior to the actual testing of sera an emulsion was prepared by adding (with constant stirring) 1 c.c. of the stock ether solution to 35 c.c. of freshly distilled water which had been warmed to 65 or 70° C. This mixture was then heated slowly to boiling and allowed to simmer until the final volume reached 30 c.c. After cooling to room temperature, the emulsion was ready for use. That which was not used within a few hours was discarded.

One cubic centimeter of this emulsion was placed in a centrifuge tube containing 0.2 c.c. of the patient's serum diluted with 4 c.c. of physiologic saline (0.85 per cent sodium chloride solution). The mixture was shaken thoroughly, stoppered with cotton, and permitted to stand undisturbed at room temperature for twenty-four hours. The reactions were graded in terms of negative and 1 to 4 plus, a 4 plus indicating complete flocculation which left the supernatant fluid water-clear. A control tube containing 4 c.c. of saline and 1 c.c. of emulsion without serum was always made to test the stability of the emulsion.

It is essential that all glassware be scrupulously clean. If sera are permitted to stand for six hours or longer, false positive reactions will occur with normal sera and increasingly positive reactions with abnormal sera. Knowing that this invariably occurs, we have dispensed with forty-eight-hour readings.

## RESULTS

The sera of 178 patients with known diffuse parenchymatous liver disease brought about flocculation of the cephalin-cholesterol emulsion in 173 instances (Table I). Only five (or 2.8 per cent) were read as negative reactions. Two of these occurred in the presence of cirrhosis which anatomically was early but nonetheless definite. Another occurred in the presence of carcinoma of the liver and a fourth in the presence of diffuse atrophy of the liver. The fifth negative test was obtained in a late case of catarrhal jaundice; it is felt that the test would undoubtedly have been positive at an earlier time.

TABLE I  
KNOWN LESIONS OF THE LIVER—DIFFUSE

NUMBER OF CASES	DIAGNOSIS	NEGATIVE		POSITIVE		
		0	1 PLUS	2 PLUS	3 PLUS	4 PLUS
127	Cirrhosis	2	0	4	48	73
32	Catarrhal jaundice	1	0	3	7	21
2	Carcinoma of liver	0	1	0	0	1
8	Acute hepatitis	0	0	0	2	6
5	Atrophy of liver	0	1	0	2	2
2	Thrombosis of the portal vein	0	0	0	0	2
1	Hand-Christian-Schüller disease	0	0	0	0	1
1	CCl <sub>4</sub> poisoning	0	0	0	1	0
Total 178		3	2	7	60	106
100 per cent		1.7	1.1	3.9	33.7	59.6
	Negative, 2.8 per cent					
	Positive, 97.2 per cent					

Discrete lesions of the liver, involving only a small part of the parenchyma, were associated with a significant flocculation in 53.3 per cent of the instances (Table II). Attention was usually, but not always, directed to these lesions by

\*We wish to express our thanks to Dr. David Klein, of the Wilson Laboratories, who so generously furnished these mixtures to us.

the presence of obstructive jaundice. There was, however, no correlation between the presence, duration, or severity of the jaundice in these cases and the presence or absence of flocculation. The positive reaction in the patient with liver abscess may or may not be significant, for a pyrogenic process anywhere in the body, as will be emphasized later, may produce a positive test.

TABLE II  
KNOWN LESIONS OF THE LIVER—DISCRETE

NUMBER OF CASES	DIAGNOSIS	NEGATIVE		POSITIVE		
		0	1 PLUS	2 PLUS	3 PLUS	4 PLUS
40	Carcinoma (metastatic or direct extension into liver or bile ducts)	17	3	9	5	6
3	Cysts of liver	0	0	0	3	0
1	Abscess of liver	0	0	1	0	0
1	Bullet wound of liver	1	0	0	0	0
Total 45		18	3	10	8	6
100 per cent		40.0	6.7	22.2	17.8	13.3
	Negative, 46.7 per cent					
	Positive, 53.3 per cent					

In Table III are grouped those cases in which a reasonable suspicion of dysfunction of the liver existed, even though in some instances there may have been no demonstrable anatomical lesion of the liver. Of these 180 cases, sixty-seven were associated with definite flocculation (37.2 per cent). Cholecystitis and cholelithiasis were responsible for twenty-one of these, chronic passive congestion for twenty-four, and chronic alcoholism for eight. No correlation could be established between the duration of cholecystitis and cholelithiasis and the presence or absence of flocculation. Negative reactions were encountered in patients whose history extended over a fifteen- to twenty-year period and positive reactions in patients with histories of only one to three months' duration. The diagnosis of cardiac cirrhosis is not warranted on the basis of a positive flocculation, for autopsy findings have not been confirmatory. The possibility of detecting early cirrhosis among patients suffering from alcoholism has been studied carefully. Although 20 per cent of such patients had a significant flocculation, none has returned with clinical cirrhosis even though two to three years have lapsed in some instances.

TABLE III  
SUSPECTED OR PROBABLE DYSFUNCTION OF THE LIVER

NUMBER OF CASES	DIAGNOSIS	NEGATIVE		POSITIVE		
		0	1 PLUS	2 PLUS	3 PLUS	4 PLUS
44	Cholecystitis and cholelithiasis	20	3	6	8	7
40	Chronic alcoholism with varying degrees of icterus and hepatomegaly	27	5	1	4	3
55	Cardiac disease with chronic passive congestion	24	7	8	8	8
4	Congenital hemolytic icterus	2	0	0	0	2
16	Eclampsia	5	5	1	3	2
18	Diabetes mellitus	12	0	2	1	3
3	Hyperthyroidism	3	0	0	0	0
Total 180		93	20	18	24	25
100 per cent		51.7	11.1	10.0	13.3	13.9
	Negative, 62.8 per cent					
	Positive, 37.2 per cent					

A control series of 105 tests is summarized in Table IV. Those from subjects without known disease (interns, residents, and laboratory technicians) were negative in all instances. Flocculations thought to be significant did occur, however, in nineteen patients who were not suspected of having any liver disease. Twelve of the nineteen positive reactions occurred in the presence of infection. This has been noted repeatedly, but unfortunately not all instances are sufficiently clear cut to permit inclusion in this paper. It has become the custom in this laboratory to disregard positive reactions in the presence of infectious disease except as a possible indication of transient dysfunction to be expected with other systemic diseases. If one corrects the percentage of positive reactions accordingly, there remain 3.9 per cent of unpredictable and unaccountable "false positives."

TABLE IV  
NO KNOWN LESION OR DYSFUNCTION OF THE LIVER

NUMBER OF CASES	DIAGNOSIS	NEGATIVE		POSITIVE		
		0	1 PLUS	2 PLUS	3 PLUS	4 PLUS
61	Normal (interns, etc.).	59	2	0	0	0
3	Pernicious anemia	3	0	0	0	0
6	Tuberculosis	4	0	0	2*	0
1	Mesenteric artery thrombosis	0	0	0	1	0
4	Pancreatitis	3	0	0	1	0
2	Leucemia	2	0	0	0	0
3	Malaria	1	0	0	1	1
1	Constrictive pericarditis	1	0	0	0	0
1	Boeck's sarcoid	0	0	1	0	0
1	Pyelonephritis	0	0	0	0	1
6	Glomerulonephritis	4	1	0	1	0
1	Renal dwarfism	0	0	0	1	0
6	Lobar pneumonia	3	0	0	2	1
9	Abscess formation	3	0	3	1	2
Total 105		83	3	4	10	5
100 per cent		79.0	2.9	3.8	9.5	4.8
	Negative, 81.9 per cent					
	Positive, 18.1 per cent					

\*Complicated by empyema or lung abscess

It is desirable to check further the accuracy of the test by comparison with other hepatic function tests. In Table V is indicated the percentage of positive tests obtained with the cephalin-cholesterol flocculation test, the bromsulfalein test,\* the intravenous hippuric acid test,† and the serum diastase test.‡

TABLE V  
COMPARISON OF CEPHALIN-CHOLESTEROL FLOCCULATION TEST WITH OTHER LIVER FUNCTION TESTS

NUMBER OF CASES*	DIAGNOSIS	PER CENT POSITIVE TESTS			
		C.C.	I.V. HIP.P.	B.S.P.	DIASTASE
127	Cirrhosis	98.5	83.1	89.1	62.3
32	Catarrhal jaundice	96.8	58.3	50.0	56.6
44	Cholecystitis and cholelithiasis	47.7	66.7	---	44.8
40	Chronic alcoholism	20.0	35.7	66.7	34.3
40	Carcinoma of liver or biliary tract	50.0	100.0	100.0	65.3

\*All four tests were done in at least 75 per cent of the cases.

\*Mateer's modification of the Rosenthal test was used. A single blood specimen was removed forty-five minutes following the injection of 5 mg. of the dye per kilogram of body weight. Any retention was considered abnormal.

†A urine specimen was collected exactly one hour following the injection of 1.77 Gm. of sodium benzoate (equivalent to 1.5 Gm. of benzoic acid). Excretion of less than 1.0 Gm. of hippuric acid (equivalent to 0.80 Gm. of sodium benzoate) was considered abnormal.

‡A diastase level of 60 Somogyi units or less was considered abnormal.<sup>3</sup>



With diffuse parenchymatous liver disease, the cephalin-cholesterol test surpassed the other tests used in the frequency with which supposed hepatic dysfunction was detected. On the other hand, the intravenous hippuric acid and the bromsulfalein tests both gave more frequent indication of liver disease in the presence of discrete or focal lesions of the liver.

A decrease in serum albumin with a compensatory increase in globulin is frequently associated with liver disease.<sup>16</sup> Obviously, loss of albumin as in renal disease or increase in globulin with chronic infection will result in similar serum protein patterns. An effort has been made therefore to collect instances of all sorts with disturbances of albumin-globulin ratios. Almost all cases of cirrhosis had albumin-globulin ratios of less than 1:4. Since these cases form such a large part of this series, it is not surprising that 89.5 per cent of the 124 with low ratios had positive flocculations. It is striking, however, that 10.5 per cent of these had negative cephalin-cholesterol flocculation tests, indicating that relative increases in the globulin fraction are not necessarily associated with a positive flocculation. Conversely, 45.4 per cent of those with "normal" ratios had positive flocculations indicating that increase in the globulin fraction need not occur or may be very slight in the presence of a positive test.

An interesting observation, not included in Tables I to V, has to do with the flocculation test in pregnancy and in the newborn infant (Table VI). In studying jaundice of the newborn infant, it was noted that 66.7 per cent of fifteen such infants had positive flocculation tests. The sera of eleven nonjaundiced

TABLE VI  
CEPHALIN-CHOLESTEROL FLOCCULATION TEST IN MOTHERS AND NEWBORN INFANTS

NUMBER OF CASES	DIAGNOSIS	NEGATIVE			POSITIVE		% NEGATIVE		% POSITIVE	
		0	1 PLUS	2 PLUS	3 PLUS	4 PLUS				
15	Newborn infants (jaundiced)	1	4	7	3	0	33.3		66.7	
11	Newborn infants (not jaundiced)	4	2	2	3	0	54.6		45.4	
9	Normal pregnancy (six to nine months gestation)	9	0	0	0	0	100.0		0	
65	Normal pregnancy (at term plus or minus one or two days)	37	5	10	12	1	64.6		35.4	

newborn infants also brought about flocculation of the emulsion almost as frequently (45.4 per cent). In all these infants, the flocculation test became negative in from seven to ten days. The sera of approximately one-third of normal pregnant women produced flocculation of the emulsion from twenty-four to forty-eight hours prior to delivery and continued to do so for from seven to ten days following parturition. In twelve of thirteen instances studied, the degree of positivity in mother and infant ran a parallel course. More detailed information concerning this phenomenon has been reported elsewhere.<sup>15</sup>

#### DISCUSSION

The data presented are in general agreement with those published by other investigators and therefore confirm further the usefulness of the cephalin-cholesterol flocculation test (Table VII). Its maximum value is apparent in

acute hepatitis, catarrhal jaundice, cirrhosis, or any other diffuse parenchymatous disease of the liver. A negative reaction, however, does not exclude one of these lesions. Similarly a positive flocculation cannot be interpreted as necessarily indicating liver dysfunction in the presence of other systemic disease.<sup>1, 6, 11</sup> As with flocculation tests for syphilis, further observation may clarify those situations in which "false positives" may be encountered. The data presented suggest that in the presence of infection, or during the puerperium, a positive test may be encountered without other evidences of liver dysfunction.

TABLE VII

COMPARISON OF PERCENTAGE OF POSITIVE CEPHALIN-CHOLESTEROL FLOCCULATION TESTS OBTAINED BY VARIOUS INVESTIGATORS

	CLAY AND MOORE <sup>1</sup>	HANGER <sup>3, 4</sup>	KIESCHNER AND GLICKMAN <sup>5</sup>	LIPPMAN AND BAKST <sup>6</sup>	NADLER <sup>10</sup>	POHLE AND STEWART <sup>11</sup>	ROSENBERG <sup>13, 14</sup>	WADE AND RICHMAN
Acute hepatitis	---	---	65.5	---	100.0	94.4	100.0	100.0
Catarrhal jaundice	100.0	100.0	---	100.0	100.0	---	---	96.9
Cirrhosis	95.0	78.3	70.6	83.4	---	100.0	81.8	98.4
Congestive failure	20.0	0	---	40.0	---	37.9	22.3	44.7
Hemolytic icterus	---	0	33.3	---	---	---	---	50.0
Normal	0.2	0	---	4.8	---	0	---	0
Obstructive jaundice	---	11.7	17.3	22.1	3.6	78.3	---	48.7

Indeed, the purely empirical nature of the cephalin-cholesterol flocculation test is one of its chief disadvantages. Hanger believes the mechanism of the test to be similar to that of the flocculation tests for syphilis and to be dependent upon an altered globulin component<sup>3, 4</sup>. Quantitative as well as qualitative changes in the serum proteins encountered in nephrosis, sarcoidosis, chronic empyema, etc., are not necessarily associated with flocculation. The parallel course run by the flocculation test in mother and newborn infant suggests that the responsible agent does pass the placental barrier. The occurrence of positive tests in the presence of infectious disease as well as in the presence of allergic manifestations\* lends some credence to Hanger's hypothesis. Until some definite rationale can be established, the test will probably be of limited usefulness.

Failure to understand the mechanism of the test could readily be forgiven if accurate distinction between obstructive and nonobstructive jaundice were possible, as has been claimed by several observers.<sup>3-5, 14</sup> The experiences in this laboratory are more akin to those of Pohle and Stewart,<sup>11</sup> who were unable to confirm this contention. It is indeed reasonable to expect a negative reaction until such time as obstruction can bring about secondary liver damage, but strongly positive flocculations have been encountered during the early phases of such obstruction and negative reactions after many years of repeated and severe bouts of obstructive disease. Sera of 48.7 per cent of the patients with obstructive jaundice caused flocculation of the emulsion, while the sera of 84.3 per cent of the patients with nonobstructive jaundice produced a positive re-

\*Positive reactions have been encountered in the presence of asthma and urticaria, but too few instances in which liver disease can be excluded have been studied to justify description in this report.

action. Obviously one could not argue convincingly in any single instance concerning the nature of the jaundice.

Variations in the degree of positivity which occurred during the course of catarrhal jaundice as well as during the treatment of cirrhosis tend to confirm the claim that the flocculation test is of prognostic value.<sup>3, 4, 14</sup> These data will be reported elsewhere.

Conflicting reports have been published concerning the correlation of the cephalin-cholesterol flocculation test with other liver function tests.<sup>4, 11</sup> As is to be expected, however, one test appears more useful in one situation than does another. No exact correlation is demonstrable. The bromsulfalein test (Mateer's modification) in the absence of jaundice gives the most consistent evidence of liver disease. The intravenous hippuric acid and cephalin-cholesterol flocculation tests are next most reliable. The serum diastase test is least valuable of the tests examined.

#### CONCLUSIONS

1. The cephalin-cholesterol flocculation test is positive in 97.8 per cent of patients with diffuse parenchymatous liver disease.
2. Lesser percentages of positive reactions are encountered in focal disease of the liver.
3. Negative reactions may be encountered in the presence of minimal lesions but usually indicate the absence of hepatic dysfunction.
4. "False positives" occur during the course of infections, in the presence of allergic disease, or during the puerperium or the neonatal period.
5. No particular value can be ascribed to the test in the differentiation of obstructive from nonobstructive jaundice.
6. Quantitative changes in the globulin have no apparent role in determining the occurrence of positive reactions.

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# THE VALUE OF THE WELTMANN SERUM COAGULATION REACTION FOR THE DIAGNOSIS OF CERTAIN FORMS OF MALIGNANT NEOPLASTIC DISEASE

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WELTMANN, in 1930,<sup>1, 2</sup> described a simple serum reaction which shows characteristic changes in various pathologic clinical conditions. If normal blood serum is diluted fifty times with a solution of  $\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$ , the concentration of which ranges from 0.1 to 0.01 Gm. per 100 c.c., and is then boiled for fifteen minutes, it will coagulate in those tubes containing the 0.1 to 0.4 or .05 per cent concentrations (first 6 or 7 tubes). This constitutes a normal serum coagulation reaction or a normal coagulation band. Under abnormal conditions coagulation may occur in fewer than 6 test tubes (the coagulation band is shortened or shifted to the left) or there may be coagulation in more than 7 test tubes (the coagulation band is lengthened or shifted to the right). Shortening of the coagulation band is found in exudative inflammation or in processes which lead to tissue necrosis in the body. The change is independent of the cause of this necrosis. It is also found in nephrosis. Lengthening of the coagulation band is found in processes which lead to fibrosis, particularly cirrhosis of the liver, and in liver damage, hemolytic anemias, and septicemias.

The present investigation was undertaken to determine whether this reaction could help in the detection of malignant neoplastic disease in the body and whether it could help differentiate between similar clinical conditions; for example, between a new growth and other pathologic changes. Theoretically, the coagulation band would not be changed by the presence of the tumor itself but would be altered by regressive or inflammatory changes present in the tumor tissue.

*Method.*—The Weltmann test was performed according to Weltmann's original method. It is, however, advantageous to use calcium chloride containing 2 molecules of water of hydration as suggested by Dees<sup>3</sup> instead of the very hygroscopic product containing 6 molecules of water.<sup>4</sup> A lengthening of the coagulation band to tube 7½ is abnormal, although it has been shown that this might occasionally occur in old people without obvious reason.<sup>5</sup> Sera from normal individuals show coagulation in 6 to 7 test tubes, as stated by Weltmann and many other investigators. Kraemer,<sup>6</sup> however, found that sera from normal individuals gave a coagulation band of from 5 to 7 tubes. Although the presence of some kind of infection is the probable reason for the value of 5 in apparently normal individuals, we shall in this paper designate a coagulation from 0 to 4 as significantly shortened; a coagulation of 5 is regarded as being suggestively shortened.

*Results.*—Sera of 128 patients with definite evidence of malignant neoplastic disease were examined. In Table I the cases are grouped according to

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the organs in which the neoplasm originated. Exception is made for the last three groups which include malignant tumors combined with obstructive jaundice, malignant tumors in which ascites was the predominant clinical feature, and malignant tumors in which involvement of the bones was present.

In the control group, sera from 300 patients with various diseases were examined. Many of these sera were taken from patients who had lesions which clinically were similar to malignant neoplastic lesions; for example, fibrosis of breast tissue, fibromyomas of uterus, etc.

TABLE I

ORGAN	NUM- BER OF CASES	COAGULATION IN NUMBER OF TEST TUBES								
		0	1 (0.067 % CaCl <sub>2</sub> · 2H <sub>2</sub> O)	2 (0.0603 % CaCl <sub>2</sub> · 2H <sub>2</sub> O)	3 (0.0536 % CaCl <sub>2</sub> · 2H <sub>2</sub> O)	4 (0.0469 % CaCl <sub>2</sub> · 2H <sub>2</sub> O)	5 (0.0402 % CaCl <sub>2</sub> · 2H <sub>2</sub> O)	6 (0.0335 % CaCl <sub>2</sub> · 2H <sub>2</sub> O)	7 (0.0268 % CaCl <sub>2</sub> · 2H <sub>2</sub> O)	7½ (0.0234 % CaCl <sub>2</sub> · 2H <sub>2</sub> O)
Brain	1					1				
Larynx	1			1						
Thyroid	1					1				
Lung	8	1		1	1	1	3	1		
Uterus	3					2	1			
Cervix	4				1		2		1	
Ovary	2	1		1						
Kidney	4			1	1	1	1			
Esophagus	4				1	2	1			
Stomach	16	1		5	1	2	6	1		
Ileum	2				2					
Colon	19	2	1	3	3	3	3	1	3	
Rectum	11		1	1		4	4	1		
Breast	9			1	1	1	3	1	2	
Gall bladder	1							1		
Carcinomatosis and ascites	11	5	1	1	2	1			1	
Cases with bone involvement	12	8	1	1		1	1			
Cases with ob- structive jaundice	19					3	4	5	3	4
Total number	128	18	4	16	13	23	29	10	11	4

Among the 128 patients suffering from malignant neoplastic disease, twenty-one (16.4 per cent) showed a normal coagulation band, four (3.1 per cent) a lengthened, twenty-nine (22.7 per cent) a suggestive, and seventy-four (57.8 per cent) a markedly shortened coagulation band.

Among the nineteen patients in whom obstructive jaundice was the prominent clinical feature, ten had carcinoma of the pancreas and three each had carcinoma of the gall bladder, stomach, or bile ducts. Only three of these patients had a coagulation band of 4 and four a coagulation band of 5; the others had a normal or slightly lengthened coagulation band. Jaundice and hepatic damage tend to lengthen the coagulation band and to counterbalance the tendency to the left shift which might be initiated by the presence of the tumor. The coagulation band in these patients is therefore frequently normal or slightly lengthened. If in a case of jaundice the coagulation band is shortened, diffuse hepatitis, which shows marked right shift of the coagulation band, can be excluded with a good degree of probability. No help, however, is gained by the test concerning the etiology of the obstructive jaundice, since inflammatory changes may accompany obstructive jaundice caused by gallstones as well as by carcinoma.<sup>4</sup> If the jaundice cases are excluded, a left shift in the coagula-

tion band was found in about 88 per cent of 109 sera. Shortening was marked in seventy-one sera (65.2 per cent). The value of the reaction in the diagnosis of malignant neoplastic disease, however, is markedly diminished if the differential diagnosis of diseases which resemble each other clinically is considered. In patients with infiltrative lesions in the lung, the coagulation band will be shortened regardless of the underlying disease, whether carcinoma, exudative tuberculosis, or pneumonitis. The coagulation band, however, will be lengthened if the infiltration is due to a fibrosing process in the lungs. The reaction is of little diagnostic help in carcinoma of the breast and the female genital organs, since patients with inflammatory lesions or benign tumors, that is, fibromyomas with necrosis, may show a left shift in the coagulation band. This is equally true for the differentiation between benign fibroadenomatous and carcinomatous enlargement of the prostate. In carcinomas of the gastrointestinal tract, however, a positive reaction is of diagnostic value. In patients with symptoms referable to the esophagus, a shortened coagulation band favors the diagnosis of an organic lesion as opposed to spasm. If disease of the stomach is suspected, a shortening of the coagulation band suggests the presence of carcinoma or ulcer in an active stage. In cases of hematemesis a shortened coagulation band is evidence against esophageal varices as the cause of the gastric bleeding, since in the most common disease leading to this complication, cirrhosis of the liver, there is usually a lengthened coagulation band. In the presence of hyperchromic anemia, a normal or lengthened coagulation band supports the diagnosis of pernicious anemia, while a shortened coagulation band points to a complicating carcinoma.

If the bones are involved by neoplastic disease, very marked shortening of the coagulation band is found. Among twelve patients, five primary tumors were in the prostate, three in the breast, one in the kidney, and one in the cervix; one patient had multiple myeloma, and in another the primary site of the carcinoma could not be determined. We found a Weltmann reaction of 0 in eight of nine patients in whom there was marked involvement of the bones.

The coagulation band was found to be of aid in the differential diagnosis of conditions associated with ascites. If in a case of this kind the ascites is not a complication of renal or cardiac disease or due to tuberculosis, the most important differential diagnosis rests between carcinomatosis of the peritoneum and cirrhosis of the liver. Of eleven patients examined, ascites was due to carcinomatosis of the ovary in ten and to an undetermined neoplasm of the intestinal tract in one. The coagulation band was 0 five times, 1 to 4 five times, and normal only once. In a case of subleukemic lymphatic leucemia, in which ascites was the leading clinical symptom, the coagulation band was 4. This was the first laboratory examination which lead to reconsideration of the diagnosis of cirrhosis of the liver. In cirrhosis of the liver, on the other hand, the coagulation band is usually lengthened. In nine patients in whom ascites was the predominant clinical symptom, we found coagulation bands of 8 four times, of  $7\frac{1}{2}$  twice, and of 7 twice; only once, in a case complicated by severe inflammatory pelvic disease, proved by autopsy, was a coagulation band of 5 or 6 found.

#### DISCUSSION

The diagnostic and prognostic significance of changes in the coagulation band in different diseases has been established in a great number of cases by various authors.<sup>4-13, 15-22</sup>

The coagulation band is a nonspecific reaction. Both lobar pneumonia and nephrosis, for instance, will lead to a marked left shift. The test is useful, therefore, only when certain special diagnostic or prognostic problems are involved. This has been stressed very strongly by Teuffl<sup>7</sup> and Levinson and Klein.<sup>11</sup> Two diseases present at the same time may influence the coagulation band in the same direction or counterbalance each other. It is therefore, necessary to define the differential diagnostic problem before employing the test. If this is done, it can be of considerable diagnostic value.

The changes of the coagulation band in patients having malignant neoplastic diseases have been investigated by several authors. Some<sup>23, 24, 25</sup> did not find significant changes, while others<sup>7, 12, 13, 16, 26-29</sup> found a left shift in many of the patients examined. Our results agree well with this latter result, since 65 per cent of our patients with carcinoma not complicated by obstructive jaundice showed a significant shift and an additional 23 per cent a suggestive shift to the left. The test may be applied, therefore, as a diagnostic procedure when the presence of malignant neoplastic disease is suspected. This is in full agreement with Kraemer,<sup>6</sup> who feels that any patient having a coagulation band of 4 or less has either an exudative inflammatory disease or a malignant neoplastic process. The test is very valuable in the differentiation of organic disease from functional nervous disturbance. If, however, the clinical examination reveals the presence of a lesion in lungs, breast, female genital organs, or prostate, little information is gained by this reaction, since inflammatory lesions cause the same degree of shortening of the coagulation band. Similar findings have been reported by Ottsen.<sup>28</sup> The coagulation band is of value in the diagnosis of malignancies of the stomach, especially if a pernicious anemia-like blood picture or hematemesis is the leading clinical manifestation. It is frequently of diagnostic significance in malignancies of the esophagus, as well as of the small and large intestines. The Weltmann test is of great value in the differential diagnosis in patients in whom ascites is present provided that cardiac, renal, and tuberculous disease can be excluded. Finally, in those in whom diffuse neoplastic involvement of the bones is present, the coagulation band is markedly shortened. Occasionally such shortening may be found in diseases of the bone not due to neoplastic malignant disease, for example, Paget's disease with pathologic fracture, but these are exceptions and are comparatively rare. The behavior of the coagulation band in multiple myeloma deserves some comment. In one instance we found a left shift to 0. Similar results have been reported in the literature.<sup>12</sup> Fleischhaecker,<sup>30</sup> on the other hand, described a lengthening of the coagulation band to 10 test tubes as characteristic for this disease.

A satisfactory explanation for the mechanism of this reaction was lacking until recently.<sup>3</sup> Definite progress has been made since the analysis of the serum protein fractions has been made possible by the electrophoretic procedure of Tiselius. Changes in the gamma globulin were found to be responsible for the positive result in the colloidal gold reaction in blood and liquor and for the positive cephalin flocculation test.<sup>31-33</sup> Scherlis and Levis<sup>31, 34</sup> have recently shown that sera containing much alpha globulin usually have a shortened coagulation band and those with a small amount of alpha globulin usually have a prolonged coagulation band. Thus it appears probable that changes occurring in the qualitative composition of the serum proteins produce the altered colloid stability of the serum.



## SUMMARY

The Weltmann serum coagulation reaction shows a marked left shift in the large majority of patients with malignant neoplasm. It is suggested, therefore, that the test has differential diagnostic value in selected cases of suspected malignant neoplastic disease. This is particularly true when the lesion is in the gastrointestinal tract. When, however, the lesions are in the lungs, breast, female genital organs, or the prostate, the test is of little help in differentiating malignant from similar benign neoplastic or inflammatory conditions. When ascites exists and is not a complication of previous cardiac, renal, or tuberculous disease, a shortened coagulation band is suggestive of carcinomatosis, while a lengthened coagulation band is suggestive of cirrhosis of the liver. A very marked shortening of the coagulation band is also found in the majority of patients in whom neoplastic involvement of the bones is present. An unexpected shortened coagulation band, not explained by the clinical findings, should always suggest that a thorough examination of the skeleton be made.

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TABLE II

ACUTE TOXICITY TO MICE OF QUININE AND OF ATABRINE AND FOUR OF ITS SALTS

COM- POUND	FORM	LD <sub>50</sub> ± S.E.,* (GM./KG.) ACCORDING TO ROUTE OF ADMINISTRATION			
		ORAL	SUBCUTA- NEOUS	INTRA- PERITONEAL	INTRAVENOUS
Quinine	Hydrochloride	0.880 ± 0.017	—	—	—
Atabrine	Base (in oil of sesame)	0.755 ± 0.006	—	—	—
Atabrine	Base	0.888 ± 0.023	—	—	—
Atabrine	Dihydrochloride	0.706 ± 0.016	0.840 ± 0.016	0.194 ± 0.008	0.020 ± 0.001
Atabrine	Disulfamate	—	0.900 ± 0.009	0.258 ± 0.005	0.028 ± 0.001
Atabrine	Dilactate	—	0.980 ± 0.027	0.257 ± 0.004	0.035 ± 0.002
Atabrine	Dimethane sulfonate	—	0.890 ± 0.011	0.218 ± 0.003	0.028 ± 0.002

\*S.e. indicates the approximate standard error of the LD<sub>50</sub>, estimated graphically<sup>11</sup> and expressed in the same units as the LD<sub>50</sub>.

cutaneously, intraperitoneally, and intravenously. The tests were conducted on male albino mice weighing between 16 and 32 Gm. Most of the LD<sub>50</sub> values given in Table II represent data taken on groups of from thirty to fifty mice, although as many as ninety-six mice were used in one determination. With the one exception indicated in Table II, all of the preparations were either dissolved or suspended in water. The actual levels of toxicity are clearly shown in Table II and need not be discussed in detail inasmuch as they confirm and extend the data previously published.<sup>1, 2</sup> It is noteworthy, however, that the dihydrochloride is more toxic by mouth than by subcutaneous administration. Also, for a given route of parenteral administration, the LD<sub>50</sub> of the four forms tested is approximately the same, and a ratio of 32:8:1 holds generally for the subcutaneous, intraperitoneal, and intravenous routes, respectively.

*Toxicologic Comparison of Atabrine Manufactured by American and German Processes.*—Specimens of pharmaceutical grade Atabrine prepared by three of the four possible combinations of intermediates and processes of manufacture were administered to young adult albino rats to determine whether there was any difference in the acute oral toxicity. The data given in Table III indicate that the LD<sub>50</sub> is the same for the three preparations. Atabrine manufactured by the American process from American intermediates was given both as the free base and as the dihydrochloride. The free base proved to be slightly less toxic, but the difference cannot be considered significant in view of the respec-

TABLE III

ORAL TOLERANCE OF THE ALBINO RAT (140 TO 190 GM.) TO ATABRINE OF AMERICAN AND GERMAN MANUFACTURE

PREPARA- TION	PROCESS	FORM*	NUMBER OF RATS	LD <sub>50</sub> ± S.E. (GM./KG.)
1	American process with American intermediates	Base	50	0.670 ± 0.026
2	American process with American intermediates	Dihydrochloride	64	0.628 ± 0.030
3	German process with German intermediates	Dihydrochloride	80	0.640 ± 0.021
4	American process with German intermediates	Dihydrochloride	60	0.615 ± 0.037
5	Combination of preparations 2, 3, and 4	Dihydrochloride	204	0.615 ± 0.015

\*All dosages were given on basis of anhydrous compound.

tive standard errors. There is no question of a difference in toxicity in the three samples given in the form of the dihydrochloride nor of the equality of their toxicity with that of a combination of all three.

The three specimens of Atabrine were administered orally to dogs, six dogs receiving each specimen. The dogs weighed from 8 to 21 kg. and were given 25 mg. of Atabrine per kilogram of body weight three hours prior to feeding on each of three successive days. The animals were carefully watched for three hours after dosing for the development of symptoms of intolerance and to observe whether the drug was retained. All of the animals appeared to be nauseated to some extent and Dogs 5 and 12 vomited after eating on two days. The dogs suffered from a slight to moderate anorexia so that a 5 to 10 per cent weight loss occurred due to a decreased food and water intake. Each of the animals was sacrificed twenty-four hours after the last dosage and autopsied. No gross or histologic abnormalities due to medication were demonstrable. The heart, liver, lungs, spleen, and kidneys were removed and analyzed for Atabrine. The analytic data obtained are presented in Table IV.

Included in Table IV are the data on the two dogs which vomited part of the Atabrine given. A third dog (Dog 4) showed such marked diarrhea that absorption must have been impaired. Since it is probable that this accounts for the relatively low Atabrine content in the various tissues analyzed, these data have not been included in the averages.

The analytical data present several points of interest. It is clear that the three different lots of Atabrine are stored to the same extent in all the organs examined. Hence the amounts of Atabrine recovered in these organs, expressed as the percentages of the total dose given, are the same for all three lots. The average recovery in the whole series, with the exceptions noted above, was 17 per cent of the dose given in the three-day course of medication. The comparative affinity of liver tissue for Atabrine is well demonstrated. Three-fourths of the drug recovered was found in this organ, thereby demonstrating strikingly that the liver is the body's principal depot for Atabrine.

In addition to the acute experiments reported on rats and dogs, a growth experiment was carried out on ducks. The duck regurgitates substances having a local irritant action or a disagreeable taste. As a consequence, the  $LD_{50}$  in acute experiments could not be determined on normal ducks, and the possibility of ligating the esophagus was not explored. To determine the effect on growth, groups of ten or eleven young Pekin ducks, averaging 100 Gm. in weight, were segregated in false-bottomed cages and were fed a growth mash diet in which was incorporated a concentration of 0.005, 0.025, or 0.125 per cent (calculated as the anhydrous compound) of the three specimens of Atabrine. The two lower dosages closely approximate the dietary drug levels used in routine screening tests of Atabrine against experimental *Plasmodium lophurae* infections in the duck and represent the levels which are effective from a therapeutic and curative standpoint. The medication was continued for thirty-five days. For purposes of comparison, quinine sulfate was given to similar groups of ducks at levels comparable from the chemotherapeutic standpoint.

Figs. 1, 2, and 3 represent the average weights of the ducks throughout the experiment. The growth curves for the three different Atabrine specimens

TABLE IV  
DISTRIBUTION OF ATABRINE FOLLOWING ORAL ADMINISTRATION TO DOGS

ATABRINE DIHYDRO- CHLORIDE	DOG	TOTAL DOSE (MG.)	HEART		LUNGS		LIVER		SPLEEN		KIDNEYS		ATABRINE RECOVERED (% OF TOTAL DOSE)
			A	B	A	B	A	B	A	B	A	B	
American process with German intermediates	12	825	1.3	0.18	4.1	0.50	8.2	4.54	5.1	0.23	1.1	0.09	5.5*
	14	1523	4.1	0.39	15.6	2.14	35.4	12.20	27.0	1.08	5.8	0.35	16.2
	15	578	2.9	0.40	12.5	1.90	38.6	16.70	18.7	0.55	5.8	0.48	20.2
	60	675	3.7	0.33	17.5	2.50	33.6	15.70	17.1	0.69	15.0	0.91	20.0
	23	950	1.5	0.15	7.6	1.10	10.0	6.00	10.3	0.33	3.8	0.29	8.0
	16	600	5.5	0.67	9.1	1.30	38.0	15.30	21.8	0.91	7.0	0.52	19.0
Average			3.6	0.39	12.5	1.79	31.1	13.20	19.0	0.71	7.5	0.51	16.7
American process with American intermediates	2	728	3.8	0.41	12.3	1.42	21.5	10.20	11.7	1.05	15.2	1.15	14.2
	4	930	1.6	0.18	11.0	1.32	6.3	2.70	6.2	0.93	3.5	0.23	5.3*
	5	967	1.6	0.18	10.9	1.66	30.2	13.50	14.0	0.57	3.7	0.29	16.2
	6	1013	1.4	0.17	2.8	0.34	7.6	5.50	4.0	0.27	1.8	0.16	6.5*
	9	1088	5.2	0.45	16.0	1.71	30.0	11.80	23.8	0.69	5.0	0.34	15.0
	40	555	3.8	0.38	16.3	2.30	32.2	11.90	20.0	1.10	7.1	0.45	16.0
Average			3.6	0.36	13.9	1.77	28.5	11.85	18.6	0.85	7.8	0.56	15.4
German process with German intermediates	7	645	5.8	0.59	13.6	1.45	52.5	16.00	20.4	0.95	5.8	0.37	19.4
	13	1440	5.1	0.63	11.5	1.51	46.3	19.30	18.3	0.58	13.3	0.92	22.9
	17	763	2.9	0.24	9.3	1.30	20.4	7.50	25.5	0.47	6.9	0.45	10.0
	1	645	2.4	0.21	8.8	1.10	15.9	7.35	9.5	0.21	1.8	0.14	9.0
	22	833	6.2	0.67	12.3	1.70	32.3	16.90	17.1	0.60	8.3	0.65	20.0
	21	675	6.7	0.73	22.1	4.40	46.1	21.20	21.8	0.74	14.0	1.10	28.0
Average			4.8	0.51	12.9	1.91	35.6	14.71	18.8	0.59	8.4	0.60	18.2
Average—all			4.0	0.42	13.1	1.82	31.7	13.25	18.8	0.72	7.9	0.56	16.8

A = Atabrine—mg. per 100 Gm. of organ.

B = Per cent of total dose given.

\*Dogs 6 and 12 showed emesis after two of the doses and Dog 4 showed severe diarrhea; these values have been omitted from the averages.

are indistinguishable on the two lower dosage levels. The growth curves for the groups receiving 0.125 per cent Atabrine indicate that the specimens prepared from the imported intermediates were tolerated less satisfactorily than that from domestic sources. Only two deaths resulted in the group of ten ducks receiving the latter at this level, indicating further that this product was significantly less toxic than the Atabrine prepared by either process from imported intermediates. The odds are more than 100:1 against observing by chance six or more deaths in a group of ten when the true expectancy is only two. As may be noted from Figs. 1 and 2, six and eight deaths, respectively, were observed in the two groups receiving Atabrine from imported intermediates.

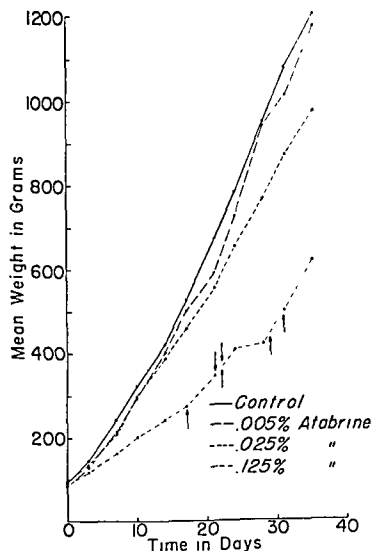


Fig. 1.—Influence of Atabrine on the growth of ducks; German process with German intermediates.

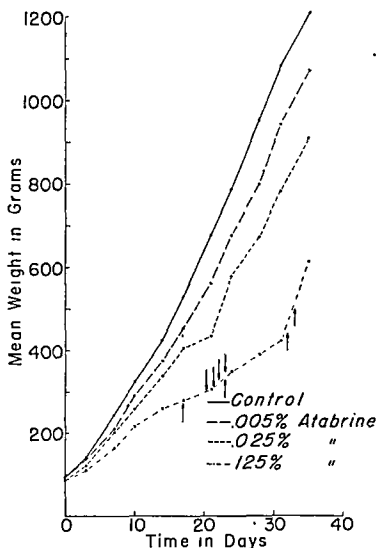


Fig. 2.—Influence of Atabrine on the growth of ducks; American process with German intermediates.

The results obtained in the parallel quinine sulfate growth test are presented in Fig. 4. Since on a weight basis quinine is only one-half as effective as Atabrine in clearing experimental *Plasmodium lophurae* infections in ducks, the concentrations of quinine used were twice those chosen in the Atabrine diets. The growth curves for the three levels of quinine agree very well with the corresponding curves for the Atabrine of American process and American intermediates, except that two deaths occurred on the highest concentration of the Atabrine.

The toxicologic data thus obtained on three species of animals show conclusively that if there is any demonstrable difference in these specimens of

Atabrine, the advantage is in favor of that manufactured by the American process from American intermediates.

*The Deposition and Retention of Atabrine in the Tissues of the Rat.*—To determine the distribution of Atabrine deposited following a single large dose, male rats weighing from 400 to 450 Gm. were fasted eighteen hours and then given 675 mg. per kilogram of body weight by stomach tube. The survivors, constituting about 40 per cent of those given this dose, were sacrificed in groups of three to nine rats at 4, 8, 24, 48, 72, 120, and 240 hours after dosing. The organs and gastrointestinal contents listed in Table V were removed and analyzed for their Atabrine content. In actual amount, the liver showed more

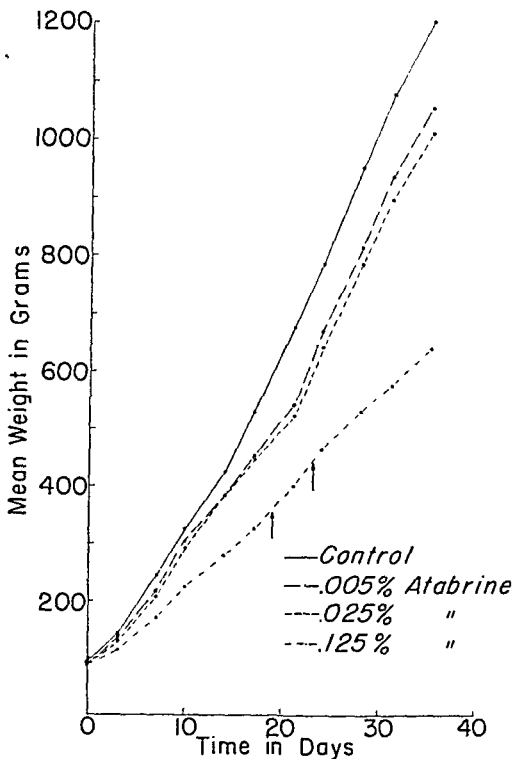


Fig. 3.—Influence of Atabrine on the growth of ducks; American process with American intermediates.

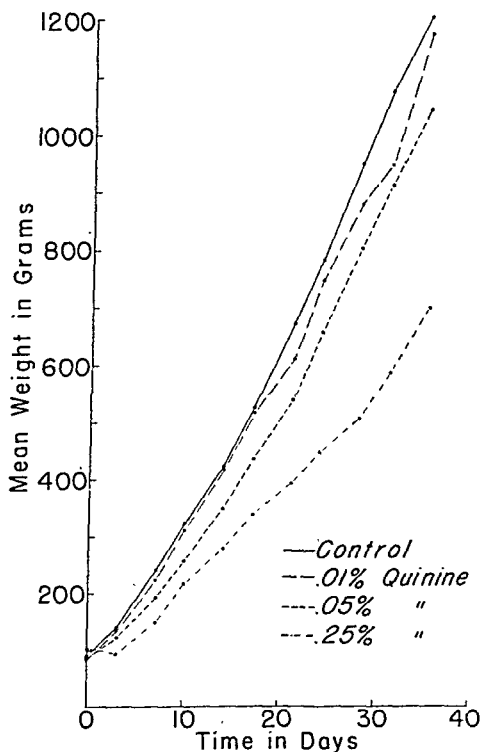


Fig. 4.—Influence of quinine on the growth of ducks.

than that found in all the other organs examined; the total amount recovered, however, was but a fraction of that given. The data indicate that the Atabrine concentration of the liver and spleen increases during the first eight to twenty-four hours and then decreases. The rate of disappearance is not the same for all organs, the indication being either that the mobilization of the deposits in the liver and spleen tends to keep the other tissues saturated or that the latter have a greater relative affinity for the drug.

*Deposition of Atabrine in Various Organs During and After Chronic Medication.*—To determine the degree of Atabrine deposition, without disturbing the course of the experiments illustrated in Fig. 1, a second experiment was

TABLE V

AMOUNT AND CONCENTRATION OF ATABRINE IN RATS AFTER A SINGLE DOSE OF 675 MG. PER KILOGRAM ORALLY.

ORGANS	AMOUNT (MG.)							CONCENTRATION (MG./GM.) OF TISSUE OR GASTROINTESTINAL CONTENTS							
	HOURS AFTER MEDICATION							HOURS AFTER MEDICATION							
	4	8	24	48	72	120	240	4	8	24	48	72	120	240	
Liver	1.81	2.45	1.65	1.31	0.70	0.75	0.52	0.13	0.16	0.10	0.08	0.04	0.04	0.03	
Kidneys	0.22	0.19	0.26	0.18	0.14	0.11	0.04	0.07	0.07	0.07	0.06	0.05	0.04	0.01	
Lungs	0.21	0.19	0.18	0.12	0.12	0.10	0.03	0.08	0.08	0.06	0.05	0.04	0.03	0.01	
Spleen	0.39	0.01	0.13	0.10	0.06	0.06	0.03	0.09	0.16	0.22	0.19	0.12	0.09	0.04	
Heart	0.04	0.04	0.04	0.02	0.02			0.02	0.03	0.03	0.02	0.02			
Stomach (empty)	0.04	0.04	0.04	0.02	0.01			0.03	0.02	0.03	0.01	Trace			
Stomach contents	2.06	0.80	0.75	0.06				0.42	0.22	0.22	0.04				
Intestinal contents	0.80	0.53	0.39	0.15				0.21	0.14	0.08	0.05				
Cecal contents	0.55	0.39	0.33	0.11				0.12	0.10	0.05	0.02				

set up, using from 45 to 50 Gm male rats in groups of such size that from five to ten animals could be sacrificed at intervals after the daily drug dosage was discontinued. Three such groups of animals received Atabrine by stomach tube daily for forty-nine days in dosages corresponding respectively to 25, 5, and 10 per cent of the  $LD_{50}$  for rats of this age and weight. A fourth group received 20 per cent of the  $LD_{50}$  for only five days. The tissue Atabrine analyses were carried out by the procedure described by Hecht<sup>1</sup>. Blood concentrations were determined by a photofluorometric method.<sup>12</sup>

The results obtained by chemical analyses of blood and liver at the indicated intervals are illustrated as means in Table VI. The data indicate that a significant blood concentration was maintained in the rat as long as Atabrine deposits existed in the liver. These deposits disappeared rapidly and approached zero by from forty to sixty days after discontinuance of medication.

TABLE VI

MEAN ATABRINE CONCENTRATION OF BLOOD AND LIVER OF RATS

DAILY DOSE (MG./KG.)	DAYS OF MEDICATION	TOTAL DOSE (GM./KG.)	NUMBER OF RATS	DAY SACRIFICED	AVE. LIVER WT. (GM.)	LIVER ATABRINE (MG./GM.)	BLOOD ATABRINE (γ/100 C.C.)
22.5	49	1.10	8	50th	9.90	0.08	25
			8	64th	11.90	0.002	3
			8	78th	11.56	Trace	Negative
			9	50th	9.15	1.62	50
45.0	49	2.20	9	61st	10.63	0.05	5
			7	78th	10.90	0.026	0-10*
			7	90th	11.71	0.004	Negative
			7	50th	12.17	4.58	300
90.0	49	4.40	8	64th	9.64	2.00	30
			7	71st	11.45	0.82	26
			5	92nd	11.47	0.106	6
			9	106th	11.83	0.007	3
180.0	5	0.90	10	6th	2.45	1.28	580
			9	13th	3.26	0.62	260
			9	20th	4.86	0.24	Trace
			9	27th	6.16	0.016	Negative
			9	34th	6.36	0.004	Negative

\*5, negative, 2, 5 to 10 γ/100 c.c.



Gross and microscopic examinations were made of the tissues of all animals in the chronic growth studies. The findings on necropsy of animals fed daily dosages of 5 per cent or less of the LD<sub>60</sub> were insignificant. However, the following description was typical of the liver of those receiving 10 per cent of the LD<sub>60</sub>.

*Liver:* Markedly enlarged, discolored, highly pigmented with Atabrine; lobules very prominent in from 25 to 30 per cent of the organ. The coloring shaded from dark reddish blue or greenish purple to dirty yellow at periphery. Two areas, 1.5 by 2 cm., were necrotic; adhesions to diaphragm, stomach, intestines, and right kidney. Large yellow masses were deposited in liver. In volume such deposits represented one-fifth to one-fourth of the entire organ.

These deposits represented necrotic liver tissue and Atabrine. However, analysis indicated that the Atabrine content of liver tissues of relatively normal gross appearance was not greatly different from that of the cheeselike yellow-green necrotic areas. The Atabrine content of grossly normal, but strongly colored, liver tissues from selected animals of those given 45 mg. per kilogram daily was 1.25 mg. per gram; the corresponding value from necrotic areas was 1.22 mg. per gram. Of those animals given daily 90 mg. of Atabrine per kilogram of body weight, the corresponding values were 2.33 and approximately 4.2 mg. per gram.

It appears, therefore, that Atabrine deposits in the liver of the rat under the conditions of these tests exist largely in the form of organic acid salts and, to a limited extent only, in the form of the base. The other typical pathologic changes were as follows:

*Kidneys:* Greatly enlarged, mottled with minute yellow granules, and deposition in tubules. Right kidney frequently adherent to liver. Left kidney free.

*Adrenals:* Enlarged about two to four times normal size and deeply pigmented with Atabrine.

*Spleen:* Very greatly enlarged. Yellow tinted.

*Heart:* Moderately enlarged and tinted yellow.

*Lungs:* Deep yellow tint.

*Bones:* Soft and yellow colored.

*Gastrointestinal Tract:* Deep yellow color.

*Skin:* Deep yellow color.

Such changes and the marked deposition of Atabrine just described largely disappeared from fifty to sixty days after discontinuance of heavy medication.

*Influence of Chronic Medication with Large Doses of Atabrine on the Blood Picture.*—Yuge<sup>13</sup> observed a marked increase in the reticulocyte count in twelve children within a week after the beginning of therapy of tropical malaria with Atabrine and Plasmochin. Steinberg<sup>14</sup> noted no morphologic or physicochemical changes in the blood after a more or less lengthy period of chronic medication with dosages within a therapeutic range. Large doses caused unimportant reductions in red cell counts and hemoglobin values, and hemolysis or formation of methemoglobin was not observed. Hecht confirmed the observations of Steinberg experimentally and also reported that the reticulo-

endothelial system of the normal dog, or of other animals, after piroplasmoses infections was unaffected by Atabrine.

Siegenbeek van Heukelom<sup>15</sup> observed that in acute tropical malaria, patients with hemoglobin values above 60 per cent showed a slight decrease and those with lesser values showed a slight improvement with Atabrine therapy. De Langen<sup>16</sup> observed that Atabrine and quinine somewhat diminished the regenerative powers of the blood system of previously barbitalized rabbits rendered acutely anemic by hemolysis or chronically anemic by blood letting.

In this study hematologic examinations (red and white cell counts, hemoglobin values, and differential counts) were made at weekly intervals on adult albino rats which were being given daily 5, 10, or 20 per cent of the acute oral LD<sub>50</sub>. No significant effect of Atabrine on the red cell, white cell, or differential counts or on hemoglobin values was noted. However, the differential counts of the medicated animals deviated slightly from the normal as indicated by an increase in the polymorphonuclear and basophilic cells at the expense of the lymphocytes. The significance of this shift is open to question.

*The Influence of Continued Ingestion of Atabrine on the Growth of Rats.*—Wright and Lillie<sup>6</sup> observed the effects of progressively increased doses of Atabrine on the growth of rats weighing from 114 to 118 Gm. and examined the vital organs for histologic changes. They observed that large doses diminished the growth rate and resulted in more or less extensive histologic changes in the liver, spleen, and other organs, such as those described, but doses of 30 mg. per kilogram (approximately 5 per cent of the acute oral LD<sub>50</sub>), given daily for one week, did not retard growth and the organic changes were either slight or absent. Because of the importance that Atabrine has assumed in replacing quinine in treating malaria, it appeared advisable to obtain additional data on this point.

Male rats weighing from 45 to 55 Gm. were maintained on a regular stock diet in individual false-bottomed wire cages. Atabrine was given six times weekly by stomach tube according to the dosage schedule shown in Fig. 5. On Saturday and Monday one-half again as large a dose was given to compensate for omitting a dose on Sunday. Groups A, B, and C each consisted of fifteen animals, whereas twenty were in Groups D and E. The doses given rats in Groups A, B, C, and D represented, respectively, 1, 2, 5, and 10 per cent of the LD<sub>50</sub> for rats of this size. Inasmuch as it has been observed that rats weighing more than 100 Gm. are more sensitive to Atabrine, the daily dosage was reduced after the twenty-first day of the experiment, when the rats had attained an average weight of about 100 Gm., in order that the daily dose would maintain approximately the same relation to the acute LD<sub>50</sub>. Twice weekly the animals were weighed and the dosage was adjusted to the new body weight. The growth curves are shown graphically in Fig. 5, in which an adverse effect on the growth of the animals in Groups C and D in comparison with that of those in Groups A, B, and E was already evident at the fourth day. At this point Groups C and D were divided and one-half of the rats in each group received 2 mol. of sodium bicarbonate for each molecule of Atabrine given thereafter.

Inspection of the growth curves indicates that the rats in Group A, receiving the lowest dosage of Atabrine, grew as well as or better than those in Group E, the controls, which grew less rapidly at first but maintained a steady rate of growth until the end of the experiment. The rats in Group B grew at a steady rate but by the end of the experiment appeared to be losing ground in comparison with the controls. The sodium bicarbonate supplements did not influence the toxicity of the Atabrine which was sufficient to retard the growth of the rats in Group C considerably and that of those in Group D markedly.

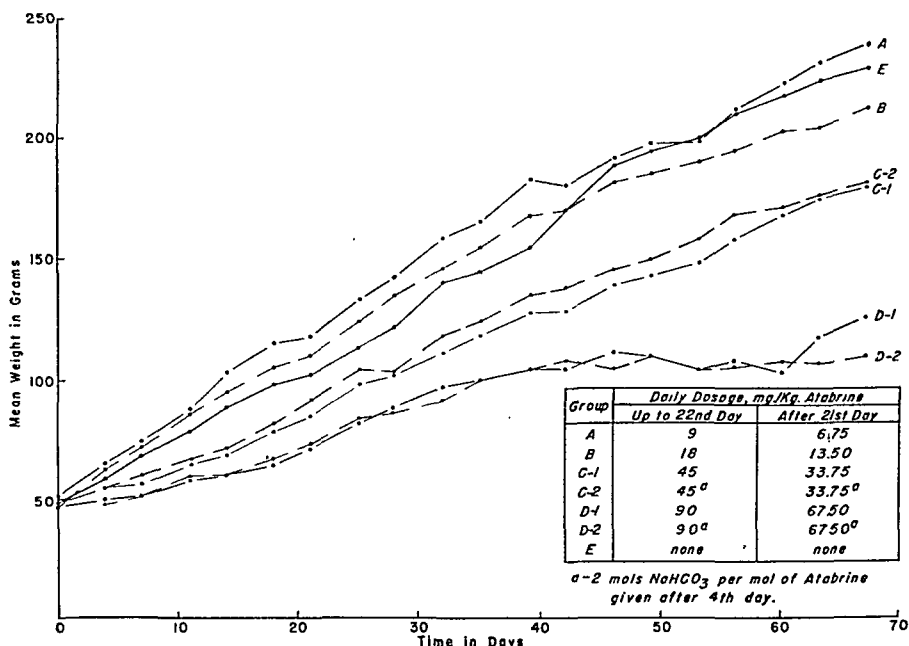


Fig. 5.—Influence of Atabrine on the growth of rats.

#### SUMMARY AND CONCLUSIONS

1. Data on the physicochemical characteristics of eight salts of Atabrine are presented.

2. When given by mouth to mice, the toxicity of quinine dihydrochloride, Atabrine base in water, Atabrine base in oil, and Atabrine dihydrochloride increases in the order named. The latter was only 25 per cent more toxic than quinine. The acute toxicity of four salts of Atabrine was determined in the mouse by three different routes of administration.

3. Atabrine manufactured by the American process from American intermediates was no more toxic to the rat than that manufactured from German intermediates by either the German or American process. The three specimens were deposited to the same extent in the tissues of dogs. While the effects of the three specimens on the growth of ducks were not different, the Atabrine from American intermediates produced significantly fewer deaths than those from German intermediates. Quinine sulfate, in doses comparable to those of Atabrine in chemotherapeutic efficacy, retarded growth to the same extent as Atabrine but did not cause any deaths.

4. Studies in which rats were given a massive dose of Atabrine by mouth show that high concentrations are reached in the tissues, and especially the liver, during the first eight hours and thereafter the amount of drug decreases, but measurable quantities still are present ten days after dosing. After forty-nine days of continuous administration of large doses, the livers of young rats showed a deposit of 4.58 mg. of Atabrine per gram of tissue and the blood level was 300  $\gamma$  per 100 c.c. Detectable amounts were still present fifty-six days later. Pathologic examination showed that the injuries to the liver and other visceral organs resulting from large doses of Atabrine healed markedly during the two months after medication was discontinued.

5. The effect on the growth of rats of daily administration of Atabrine for seventy days was determined. At levels above 2 per cent of the acute LD<sub>50</sub>, an adverse effect on growth was demonstrated. Sodium bicarbonate did not ameliorate the effect.

6. Continued administration of Atabrine had no significant effect on the blood of rats.

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# EFFECT OF THE ORAL ADMINISTRATION OF THYMOL ON EXPERIMENTALLY INDUCED TUBERCULOSIS

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IN A previous paper, Searcy, McBurney, and Rowe<sup>1</sup> published a preliminary report of the results obtained with the clinical use of thymol as a therapeutic agent in six selected cases of pulmonary tuberculosis. During the course of its clinical trial, we were engaged in determining a like effect of this agent upon tuberculosis experimentally induced in guinea pigs. The results of these investigations are set forth herein.

The report of the clinical application of thymol on human beings preceded the findings on experimentally induced tuberculosis because opportunity for clinical trial was presented before completion of the latter, which even then gave evidence of prolonging the life of infected animals. Because of successful oral administration of thymol in the treatment of actinomyces by Myers and Thienes,<sup>2</sup> Searcy and McBurney,<sup>3</sup> and Joyce<sup>4</sup> and the report by Barnes<sup>5</sup> of its use as an anthelmintic in Siam, with only two deaths among 82,000 treated individuals, no untoward results were anticipated.

In 1914, Perlman, Brown, and Raiziss<sup>6</sup> reported the use of thymol, menthol, and thymol-menthol combined as therapeutic agents against experimentally produced tuberculosis in guinea pigs infected with a virulent strain of bovine tubercle bacilli (Ravenel) by intramuscular injection of 0.4 c.c. of a 5 per cent solution in peanut oil.

Preliminary to our investigation, considerable work was entailed in the selection of the strain of organism, medium for growth, type of solvent for the thymol, and route of administration. A series of experiments, in which thymol in olive oil was administered intraperitoneally, gave rise to the formation of a profuse serous peritoneal exudate, resulting in the death of animals before evidence of tuberculous infection was established. This procedure was therefore discarded.

In all investigations the strain of *Mycobacterium tuberculosis* used was of human origin, isolated by us from the sputum of a patient with well-established pulmonary tuberculosis, and designated as strain J.\* After many trials, Holmes' medium was found best suited for growth, it being profuse and coarsely granular within three to four weeks.

Animals used were fully grown, healthy guinea pigs selected regardless of sex. Inoculations consisted of saline suspensions given subcutaneously in the inguinal region.

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Pathogenicity of the strain of *Mycobacterium tuberculosis*, using 1 mg., was determined simultaneously in eight animals; 75 and 100 per cent succumbed in six and eight weeks, respectively. Autopsy revealed gross and microscopic evidence of marked infection.

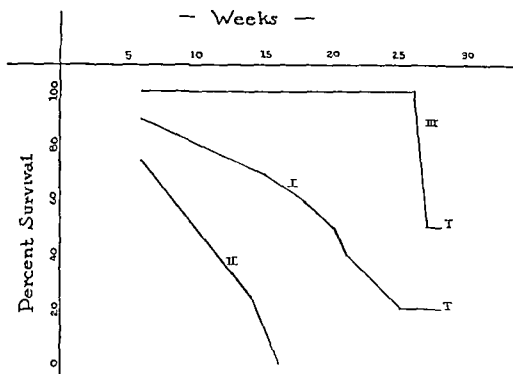
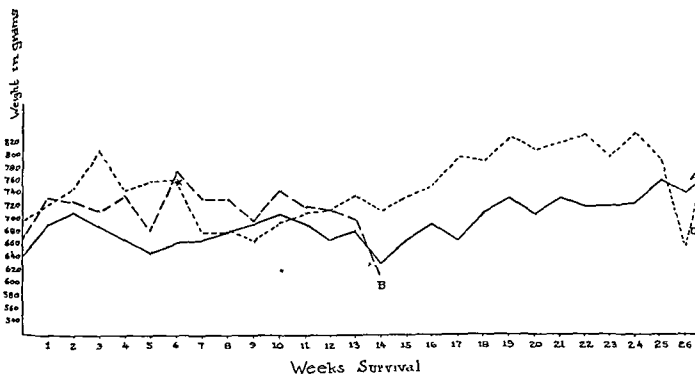


Fig. 1.—Percentage surviving at the number of weeks indicated following inoculation I, Infected; thymol. II, Infected; no thymol. III, Thymol controls.



\*One pig gave birth to two young.

Fig. 2.—Average weight curves of three groups of tested animals. A, Infected; thymol. B, Infected, untreated C, Noninfected, thymol only

Thymol medication experiments were also carried out on twelve animals receiving 1 mg. each of tubercle bacilli. Daily oral doses of thymol\* in olive oil were given in amounts which corresponded to 10 gr. per 100 pounds of body weight. This was easily administered by means of a tuberculin syringe without needle. No therapeutic effect was observed.

\*Merck's chemically pure

An experiment using a larger dose of thymol and a smaller dose of organisms was next performed. Twenty animals were arranged in three groups, I, II, and III. Those in groups I and II were inoculated with 0.0005 mg. of culture J, the amount used by Feldman, Hinshaw, and Moses<sup>8</sup> in their study of the effect of promin (sodium salt of P,P'-diamino-diphenyl-sulfone-N,N'-dextrose sulfonate) on experimental tuberculosis.

Animals in group III received no inoculation but were designated as a normal thymol control group. Thymol medication was given the pigs in this group simultaneously with those of group I; namely, one week following inoculation with tubercle bacilli. The amount of thymol used corresponded to 15 gr. per 100 pounds of body weight.

The animals in all three groups were distributed in large, well-aired, open cages as follows:

Group I (inoculated), 10 animals, thymol medication

Group II (inoculated), 5 animals, no thymol

Group III (normal thymol control), 5 animals, thymol only

All animals were well cared for, being given fresh water, oats, alfalfa hay, and fresh green vegetables daily. They were weighed one week prior to the experiment and at the end of each week during the experiment. Weight curves are shown in Fig. 2.

Results are shown in Table I and are expressed as the percentage surviving at the number of weeks indicated. This is likewise graphically portrayed in Fig. 1.

TABLE I  
PER CENT SURVIVAL OF ANIMALS IN THREE GROUPS

WEEKS FOLLOWING INOCULATION	6	14	15	18	20	21	23	25	26	27	28
PER CENT SURVIVAL											
Group I	90		70	60	50	40	30	20			20 (K)
Infected; thymol											
Group II	75	25	0								
Infected; no thymol											
Group III	*								100	50	50 (K)
Normal thymol controls; thymol only											

\*Death of one animal due to intestinal obstruction. Histologic sections showed chronic bronchitis and acute pneumonitis with no evidence of toxicity attributable to thymol.

(K), Killed.

#### GROSS PATHOLOGY

Autopsies were done on all animals. With two exceptions there was little difference in the gross appearance between the animals receiving thymol and those not receiving it. Generalized tuberculosis involving lungs, liver, spleen, mesentery, and inguinal lymph nodes was evident in the guinea pigs in groups I and II. Acid-fast stains made from lesions were positive.

Of the two exceptions, only a few small tubercles were seen in one area of the spleen in one instance and a single minute tuberculous abscess in the left inguinal region in the other. Interestingly, these two were treated animals surviving the length of the experiment and were killed at the end of the twenty-

eighth week. In view of gross findings noted in the animals in the inoculated groups, histologic sections were not made. In the pigs in group III, the thymol control group, no gross pathology was evident.

#### HISTOPATHOLOGY\*

Histologic sections were made of the tissues of the four remaining animals in group III, who received thymol only, with the following findings: In the lungs of three there was slight active hyperemia; in one, slight edema and passive hyperemia. The liver of two showed moderate fatty changes; one, severe fatty changes, and one was normal. All showed a few phagocytes containing hemosiderin in the spleen. In the kidneys of two, there was slight active hyperemia; in one, slight tubular degeneration and hyperemia of the interstitial tissue; and in another, marked cloudy swelling of the tubules. Heart and suprarenals of all four were apparently normal.

With the exception of the one animal in this group that died during the sixth week of intestinal obstruction, whose lung section showed a chronic bronchitis and an acute focal pneumonitis, the remaining four pigs showed evidence of a moderate degree of damage evidently due to the toxic action of continuous and prolonged thymol medication.

#### WEIGHT CURVES

Curves *A*, *B*, and *C* in Fig. 2 show the average weekly weights of each group of animals in the experiment. The animals in each group gained weight the first few weeks. Since environmental conditions and diet were carefully controlled, the gain may be attributed to these factors. Following this initial gain the three curves present an interesting comparison. Curve *A*, representing infected animals receiving thymol, maintains a fairly constant or level curve. Curve *B*, representing infected animals untreated, shows a decline, whereas curve *C*, representing noninfected animals receiving thymol only, steadily rises. Fluctuations or drops in each curve occur prior to death, which was preceded by a loss in weight of the animal.

#### DISCUSSION

Only 25 per cent of the animals receiving no medication (group II) were living at the end of the fourteenth week and none the fifteenth.

Of the animals in group I, receiving organisms and medication, 90, 70, 60, 50, 40, 30, and 20 per cent were living at 6, 15, 18, 20, 21, 23, and 28 weeks, respectively, following inoculation. Twenty per cent of the treated infected animals survived twenty-eight weeks, which was 86.7 per cent longer than the untreated animals receiving similar doses of the organisms. The surviving animals may have lived longer but were killed at the end of the twenty-eighth week.

When all of the infected untreated animals had died from evident tuberculous lesions, 70 per cent of the infected animals receiving thymol were living and

\*We wish to express our thanks to Dr. Jasper D. Bush and Dr. Mark C. Wheelock, Associate and Assistant Professor, respectively, Department of Bacteriology-Pathology, School of Medicine, University of Alabama, for the examination of and report on the histologic specimens, and Mrs. Irene Bush, of the Department of Pathology, for their preparation.



apparently well. At this time 100 per cent of the thymol control animals were likewise living and apparently well.

The average longevity of the animals in each of the three groups is given in Table II.

TABLE II

GROUP	DESCRIPTION	WEEKS	DAYS
I	Infected; thymol	18.7	(130.9)
II	Infected; untreated	11.6	(81.2)
III	Noninfected; thymol	27.0	(189.0)

Comparison of the figures in Table II reveals the following:

Infected animals receiving thymol lived 38 per cent longer than those receiving no thymol.

Noninfected animals receiving thymol lived 30.7 per cent longer than infected ones receiving thymol.

Noninfected animals receiving thymol lived 57 per cent longer than infected ones receiving no thymol.

Where we employed 0.0005 mg. of a human strain of the organism, Pearlman, Brown, and Raiziss<sup>6</sup> used one-tenth as much (0.00005 mg.) of a bovine strain. Three days following inoculation with the culture, they gave an initial dose of thymol intramuscularly. At five-day intervals, subsequent doses were given alternately in the right and left thighs. This was followed by a rest period of two weeks after the fourth injection. The average longevity of three groups of treated animals was ninety-two days, while that of a similar group of untreated ones was eighty-two days. The average longevity for the two methods is shown in Table III.

TABLE III  
AVERAGE LONGEVITY (DAYS)

	MC BURNEY, CASON, AND SEARCY	PERLMAN, BROWN, AND RAIZISS
Infected; thymol	130.9	92
Infected; untreated	81.2	82

While the dosage of our human strain was ten times that of the bovine strain of Perlman, Brown, and Raiziss, apparently they were equally pathogenic for guinea pigs, if the time element alone is considered.

It is of interest to note that in comparing single dosage of thymol, both correspond, being equivalent to 0.3 gr. per kilogram of body weight. Considering the variation in frequency of medication and route of administration, the average life of infected animals receiving daily oral doses in our experiments was 42 per cent longer than those receiving alternate intramuscular injections at five-day intervals.

Had we used a dosage of our human strain corresponding to the bovine strain of Perlman, Brown, and Raiziss, it is not improbable that the average life of our treated animals have been much greater. This might well be the case if experimentally produced pulmonary lesions could be treated.

An interesting and striking fact is portrayed in the weight curves (Fig. 2) with respect to the animals in groups A (infected; receiving thymol) and C

(noninfected; receiving thymol only). Both show a parallel weight gain beginning the fourteenth week, at which time medication was given every other day instead of daily. This may indicate that while thymol medication was apparently responsible for the longevity of the guinea pigs in the infected group, an even greater interval between dosage might have been more effective. Such an hypothesis forms a basis for experiments now under investigation.

It is difficult to interpret the therapeutic effects of any drug used in experimental tuberculosis in terms of the pulmonary form on human beings, since so many uncontrollable factors enter the picture.

Experimental results obtained in guinea pigs represent the effect upon a widely disseminated infection, whereas it would appear that any hope for successful medication in human pulmonary tuberculosis depends upon treating early cases. The results we obtained with thymol medication in infected guinea pigs, wherein the average life of treated infected animals was nearly fifty days longer than that of the untreated ones, might be comparable to a marked span of years in man. While it is probable that oral thymol medication in early pulmonary tuberculosis, if of any therapeutic value, may not act specifically, it might result in arresting the disease to the extent that systemic treatment and natural body defenses would become more effective if used in conjunction with the time-honored methods employed by clinicians generally. This is mere conjecture and may be determined only by well-controlled clinical trial.

While it is true that some pathologic changes occurred in normal animals receiving thymol, it was mild in degree, the kidneys being more involved than other tissues. Apparently the toxicity evidenced by thymol in these experiments is far less than that manifested by certain sulfonamide compounds, especially when dosage is not adequately controlled.

Daily doses of 5 gr. of thymol, three times daily, given throughout a period of seventeen weeks to six patients with well-advanced pulmonary tuberculosis reported by Searcy, McBurney, and Rowe<sup>1</sup> exhibited no clinical manifestations of toxicity, and repeated urine examination failed to show evidence of kidney involvement.

Later clinical reports\* on these patients showed that one died a year following treatment and another, four months following treatment from "far-advanced pulmonary tuberculosis." One was discharged home a year following treatment in "good condition" and has not been heard of since. Of the remaining three, fifteen months following treatment the disease was diagnosed "arrested" in two and "probably arrested" in the other. These cases were too few and the disease too far advanced to form a basis of conclusion. Hinshaw and Feldman<sup>9</sup> caution against hasty clinical evaluation of any therapeutic agent in human tuberculosis. They call attention to the tendency of many forms toward steady improvement without treatment. With equal emphasis they state that equal care must be taken lest some treatment be discarded prematurely.

\*Information furnished through State Hospital, Mt. Vernon, Al. copies of reports of x-ray examination of Health, Division of

Dr. Harry S. Rowe, Searcy and who likewise submitted by the Alabama State De-

## SUMMARY AND CONCLUSIONS

From the foregoing experiments it is evident that daily oral doses of 10 gr. of thymol per 100 pounds body weight produce no inhibitory effect upon massive doses (1 mg.) of virulent human strains of *Mycobacterium tuberculosis* in guinea pigs. However, where 0.0005 mg. of the organism is employed, 15 gr. of thymol per 100 pounds of body weight exert a marked inhibitory action as indicated by prolongation of the average life of animals so treated, nearly fifty days longer than those untreated.

When infected and noninfected animals were given similar oral doses of thymol on alternate days, their average weight gain was greater and more constant than when similar doses were given daily.

There was very little difference in the gross lesions, produced by the organisms, between the treated and untreated animals. However, fourteen weeks following inoculation, when all control untreated animals had died from generalized tuberculosis, 70 per cent of the treated animals were living and apparently well.

The daily oral dose employed, when given concurrently to a like group of normal guinea pigs, showed no observable evidence of toxicity throughout twenty-six weeks of treatment, since 100 per cent were living and apparently well at that time. Fifty per cent died during the twenty-seventh week, the remainder being killed at the end of the twenty-eighth week.

No gross pathologic changes were observed in post-mortem examination of the animals in this group, but histologic sections gave evidence of damage in a moderate degree.

It appears that thymol or thymol combinations administered orally as therapeutic agents in tuberculosis experimentally induced in animals and in early recognized human pulmonary forms where medication is adequately controlled are worthy of further investigation and clinical trial.

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## PROGRESS

### HUMAN COMPLEMENT

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**B**ECAUSE of its unique and important role in natural immunity, and because of the practical application of its reactions to the laboratory diagnosis of disease, the humoral entity known as complement or alexin has received considerable attention from experimenters and clinicians. In the more than fifty years since its discovery, complement has been investigated by scores of workers in immunology and bacteriology, with the result that hundreds of papers have appeared in an attempt to establish a set of criteria for its adequate definition, to elucidate its constitution and the mechanisms of its various reactions, and to determine, with reference to actual physiologic conditions, its exact role in immunity.

From the functional viewpoint, complement has been defined as recently as 1939 as "comprising all of the activities of unheated serum."<sup>1</sup> This definition, to say the least, is too inclusive, because the thermolability of complement distinguishes it chiefly from the relatively thermostable antibodies. The functions of complement which have been established with some degree of certainty, excluding those ascribed to it on the basis of circumstantial evidence and coincidence, are concerned only with immunity and may be summarized broadly as follows: (1) the extracellular destruction of invading organisms or foreign cells when specific antibody is present (bactericidal action, bacteriolysis, hemolysis), and (2) together with specific antibody, the preparation of organisms or foreign substances so that these may be removed and destroyed by the phagocytic cells of the host (acceleration of opsonification). Complement may then be said to be an innate humoral factor which functions in cooperation with immune agents and with the cellular elements to destroy a variety of invading organisms and foreign substances.<sup>2, 3</sup>

It is to be noted that this definition omits the usual contrast with antibody, "complement is not increased in the course of immunization." The repetition of this statement has become habitual despite the fact that many workers have reported an increase in the complement of certain individuals suffering with infectious disease.<sup>2</sup> In this laboratory, too, such an increase has often been encountered, and until this phenomenon is better understood, it is suggested that the distinction be narrowed by saying that the augmentation of antibody in the course of immunization is more constant, of greater magnitude, and of different nature from that occurring with regard to complement.

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From a constitutional standpoint, complement was long regarded as a "physicochemical state of fresh serum." The justification for this figurative description was the argument that complement is known only by its function, though a similar justification should then have existed for the classification of certain enzymes as "physicochemical states." It is now no longer necessary to cover ignorance in this fashion, for it is firmly established that complement consists of at least four serum components, three of which have been isolated in considerably purified form.

Until recently, knowledge of complement was confined almost wholly to guinea pig complement, and several extensive reviews of the subject have appeared.<sup>1-5</sup> The present review, therefore, is concerned mainly with a discussion of the constitution, immunologic functions, and variation in health and disease of the complement occurring in human serum. Of necessity much of this review is concerned with the work performed in this laboratory.

#### THE COMPLEX NATURE OF HUMAN COMPLEMENT

As in the case of guinea pig complement, human serum complement is composed of at least four components.<sup>6</sup> Originally each of these was defined by its participation in immune hemolysis, by a method for its inactivation with respect to this reaction, or by a method for its separation from the other components. Early attempts to "split" human complement by chemical means were inconclusive,<sup>7, 8</sup> but the recent isolation of serum fractions containing single component activities provides a material basis for the definition of at least three of the components.<sup>9-11</sup>

It is to be noted that the existence of at least four components is postulated. Since it is altogether possible that the present components may be further subdivided on the basis of activity, it is well not to be dogmatic in establishing limits for the complex nature of complement. Nevertheless, it is emphasized that the four components as now conceived are adequate for the constitutional definition of complement. All four components are necessary and sufficient for the production of immune hemolysis of sensitized sheep red blood cells as well as for the immune bactericidal reaction.<sup>12</sup>

In accordance with the terminology adopted for guinea pig complement and its components,<sup>13</sup> whole human complement is designated as human C,' and the four components are designated as C'1, C'2, C'3, and C'4.<sup>6</sup>

C'1 is defined as that component which separates *completely* with the precipitate which forms when fresh human serum is dialyzed against phosphate buffer of pH 5.4 and ionic strength 0.02.<sup>6</sup> This precipitate, hereafter referred to as Fraction 1PP, also contains some of C'3 and C'4, but *no* C'2. C'2 is thus defined as that component which remains *completely* in the supernate (Fraction 1PS) after removal of the precipitate which forms in the dialysis described. Fractions 1PP and 1PS correspond functionally to the midpiece and endpiece, respectively, described for guinea pig complement.<sup>2, 4</sup>

That component which is completely and specifically inactivated or adsorbed by yeast or the insoluble carbohydrate of yeast, zymosan, is defined as C'3.<sup>6, 14</sup>

C'4 is defined as that component which is specifically inactivated by the addition of 0.20 c.c. of 0.16 M  $\text{NH}_4\text{OH}$  per cubic centimeter of human serum.<sup>6</sup>

In each of these cases, the serum or serum fraction lacking only one component is designated as a "specifically inactivated complement," indicated by the symbol  $\text{siC}'$ . Such specifically inactivated complements, used in appropriate amounts, are the analytic tools with which the investigator is able to determine the presence or absence of a given component in another serum or serum fraction. Since all four components are necessary for the production of immune hemolysis, a given serum which contains one of the four components will combine with that  $\text{siC}'$  which *lacks* that component to complete the hemolytic system. The method in which  $\text{siC}'$  is employed for the detection of the complement components is known as "specific reactivation."

In order to obtain more consistent results, we have undertaken the preparation of a standard complement and of standard specifically inactivated complements from the isolated complement components.

#### THE ASSAY OF COMPLEMENT AND ITS COMPONENTS

A given complement may be measured by its capacity for participation in any of the immunologic reactions with which it is associated, but the most consistent results are obtained with the almost universally employed method of immune hemolysis. The assay against sensitized sheep red cells has the additional advantage of being relatively simple to perform.

Many variations of the hemolytic titration have been proposed,<sup>2, 15</sup> the principal variables being time of incubation, dilution of complement, and amount of red cell substrate. However, the method most frequently employed is that in which increasing amounts of a given dilution of complement are allowed to act against constant amounts of substrate for thirty minutes at  $37^\circ \text{C}$ . Using this method, the titer of the complement may then be expressed as (a) the minimum amount of serum required to produce a beginning tinge of hemolysis (initial point titer),<sup>16</sup> (b) the amount of serum necessary to hemolyze exactly one-half of the standard unit of sensitized erythrocytes (50 per cent hemolysis titer),<sup>17, 18</sup> or (c) the minimum amount of serum required to hemolyze completely the standard unit of substrate (complete or 100 per cent hemolysis titer). The 50 per cent hemolysis titer, occurring in the range in which degree of hemolysis bears an almost linear relationship to the amount of complement, is probably the most accurate and reproducible expression of complement titer, although it is not always suitable for use in experimental work. In general, however, in a series of titrations it will be found that all three methods of expression of titer run parallel.

In 1936 Osborn<sup>2</sup> observed that the over-all titer of a given complement should be determined or limited by the titer of that component which is present in the least titer, just as the strength of a chain is limited by the strength of its weakest link. This conception was re-emphasized by Hegedüs and Greiner<sup>19</sup> and later by Heidelberger and Mayer,<sup>20</sup> the former in experiments determining the distribution of the components in the complements of various animal species and the latter in seeking to establish a correct basis on which to compare the titers of guinea pig and human complements.

On the basis of the hemolytic assay, Osborn<sup>2</sup> found the complement titers of various animal sera to fit the following scheme:

$$\text{Guinea pig} > \text{human} = \text{rat} > \text{rabbit} = \text{cat}$$

Da Costa Cruz and Penna<sup>21</sup> found that human, monkey, and dog complements were of the same order of activity. We have estimated that guinea pig serum contains about five to six times the effective complement contained in human serum and that human serum is about two to three times more active than rabbit serum.

Hegedüs and Greiner<sup>19</sup> demonstrated that midpiece (C'1) was the component of least titer, and therefore the limiting component, of guinea pig complement. They also postulated that endpiece (C'2) was the limiting component of human complement, while it has been shown in this laboratory that C'3 is present in an effective titer equal to, or less than, that of C'2.<sup>22</sup>

If a normal human complement is treated with the various testing complements (siC') at the level of *initial* hemolysis, it is found that the effective concentrations of the components as determined by the reactivation of the siC' for the hemolytic system range as follows:

$$\text{C'1} \gg \text{C'4} > \text{C'2} \leq \text{C'3}.$$

The knowledge, then, of the effective concentration of a given component must be considered in any study of complementary activity.<sup>22</sup>

#### THE FIRST COMPONENT (C'1)

C'1 of human complement has been prepared by fractional precipitation of human serum with ammonium sulfate at 1.4 M, extraction of the resulting precipitate with phosphate buffer of pH 5.4 and ionic strength 0.02, re-precipitation with ammonium sulfate between 1.0 and 1.8 M, and final re-extraction of this precipitate with the phosphate buffer.<sup>9</sup> Obtained in this manner, C'1 has been characterized as a euglobulin with an electrophoretic mobility of  $2.9 \times 10^{-5}$  in veronal buffer of pH 7.8 and ionic strength 0.1. Examined in the Svedberg oil-turbine ultracentrifuge, the C'1 preparation was shown to contain a major component which had a sedimentation constant of 6.9 Svedbergs. C'1, isolated by the methods described, constitutes from 0.6 to 0.8 per cent of the total serum protein. With the aid of Lustig and Kondritzer,<sup>23</sup> an elementary and amino acid analysis of C'1 has been obtained; these results are considered in another section of this review together with those obtained in a similar analysis of C'2.

Purified C'1 is best preserved in 4.5 per cent NaCl, or in solutions buffered to pH 6.6 and containing greater than 0.9 per cent NaCl. So dissolved, C'1 may be kept unchanged in the frozen state for at least two years. It withstands the lyophilization process and accordingly may be preserved by drying from the frozen state.

Recently, Cohn and co-workers<sup>24</sup> have reported the concentration of human C'1 in their Fraction III-2.

Immunologically, purified C'1 is inactive per se in both the hemolytic and bactericidal systems, but when combined with the other three components, it is fully active against sensitized sheep erythrocytes and sensitized *Vibrio comma*.<sup>10</sup>

#### THE SECOND COMPONENT (C'2)

Two factors have militated against the purification of C'2. In the first place, this component is a minimum component in human complement and therefore the yields tend to be poor; and in the second place, human C'2 precipitates at a much higher range of ammonium sulfate concentration than does guinea pig C'2, thus making its separation from albumin much more difficult. Nevertheless, a highly purified fraction was prepared by precipitation of human serum at pH 6.8 between 2.4 and 2.6 M ammonium sulfate.<sup>10</sup> While all preparations of C'2 obtained by this method contained about 50 per cent albumin as determined electrophoretically, they also contained a globulin with an electrophoretic mobility of  $2.6 \times 10^{-5}$  in veronal buffer of pH 7.8 and ionic strength of 0.1.<sup>25</sup> It should be remarked that guinea pig C'2 was found to be an alpha globulin containing over 10 per cent carbohydrate and having almost full C'4 activity.<sup>26</sup> The human preparation, in contrast, had about 3 per cent carbohydrate and no C'4 activity, and if it contained any alpha globulin, this latter fraction was masked by combination with another serum protein.

Again with the aid of Lustig and Kondritzer<sup>23</sup> an elementary and amino acid analysis of C'2 was secured. In comparison with the similar analysis of C'1, the results show that C'1 has a higher concentration of hydroxy amino acids (serine, threonine), tyrosine, and tryptophane. C'2, on the other hand, has a higher content of sulfur-containing amino acids (cystine and methionine) and the basic amino acids (arginine and histidine). The higher carbohydrate content of C'1 finds its expression in higher carbon, nitrogen, and humin-nitrogen values. It should be recalled that the C'2 preparation contains about 50 per cent albumin, and this is probably reflected in the higher cystine value.

In their recent studies on the fractionation of human plasma, Cohn and his associates<sup>24</sup> concentrated C'2 in their Fraction IV.

When dissolved in 0.9 per cent NaCl and buffered to pH 6.6,<sup>27</sup> C'2 may be preserved in the frozen state for at least two years. It may also be preserved by drying from the frozen state.

Purified C'2 per se is not active in immune hemolytic or bactericidal systems, but is fully active when combined with the other three components.<sup>10</sup>

If human serum is heated at 50 to 52° C. for thirty minutes, its over-all complementary activity is lost, and simultaneously C'2 disappears.<sup>6</sup> However, it is not to be inferred necessarily that C'2 is more thermolabile than C'1, for it is more probable that the heat inactivation is a function of concentration, and since C'2 is present in lesser concentration than the other protein component (C'1), its activity disappears first.<sup>22</sup>

#### THE THIRD COMPONENT (C'3)

This component has not been isolated from either guinea pig or human serum in any great degree of concentration or purification. Refractionation of Fraction 1PP (see above) at -10° C. with 30 per cent ethanol results in a precipitate



containing 70 per cent C'3 and small amounts of C'1 and C'4.<sup>28</sup> However, much remains to be done before it can be said that a purified C'3 is available, and work in this direction is in progress.

When human serum is treated with zymosan and then examined in the Tiselius apparatus, a disturbance in the gamma globulins is noted.<sup>29</sup> This may be indicative of an association of C'3 with the gamma globulins, as is the presence of most of the serum C'3 in Fraction 1PP. However, it should be remembered that the "specificity" of the zymosan for C'3 is only relative, and that often small amounts of C'1, C'2, and other proteins are adsorbed as well, and this may very well account for the observed disturbance in the Tiselius diagram.

Thus, outside of the fact that it is a minimum component in human complement,<sup>22</sup> that it is relatively unstable,<sup>27</sup> and that there are indications that it acts as an enzyme,<sup>2, 30</sup> very little is known of the chemical nature or properties of C'3.

#### THE FOURTH COMPONENT (C'4)

The fourth component, originally defined by its specific inactivation by dilute ammonia and other amino compounds,<sup>31</sup> has been postulated to be a reactive carbonyl group attached to a protein carrier.<sup>32</sup> In guinea pig complement, its persistent association with C'2 through all stages of purification inspired the conception that C'4 was a prosthetic group attached to C'2. However, in the case of human complement, C'4 has been found associated with fractions containing C'1 as well as those containing C'2<sup>6</sup> and it was not present in either purified C'1 or C'2. It must be noted, however, that C'4 associated with C'1 in Fraction 1PP was found to be very labile, whereas in association with C'2 in Fraction 1PS it was relatively stable.<sup>27</sup> The failure to find the fourth component in the purified human C'2 preparation may have been due to the destruction of C'4 in the course of the many ammonium sulfate precipitations.

Human C'4, obtained relatively free from the other components, was prepared by dialysis of Fraction 1PP (see above) against a phosphate buffer of pH 6.6 and ionic strength 0.04. The supernate which was thereby obtained constituted from 1 to 2 per cent of the total serum protein and contained 90 per cent C'4 activity in one unit along with small amounts of C'1 and C'3. These latter two components were removed by treatment with an excess of zymosan. Prepared in this manner, C'4 is best preserved by solution in phosphate buffer of pH 6.6 made isotonic with NaCl.<sup>33</sup> Like C'1 and C'2, C'4 may be preserved by drying from the frozen state.

The purified C'4 preparation is extremely sensitive to dilute alkali and amino compounds such as ammonia and hydrazine, inactivation occurring within one minute at room temperature.

#### HUMAN COMPLEMENT IN BACTERICIDAL PHENOMENA

It has been shown<sup>2, 34-36</sup> that the bactericidal and bacteriolytic effects of blood serum are dependent upon the interaction of two serum constituents, the thermolabile complement and the thermostable antibody. Recent studies<sup>12</sup> with human serum have confirmed the older findings on the functions of complement and antibody in these phenomena and, especially on the basis of the recent

knowledge of the constitution of complement, have provided new information on the particular role of human complement in the destruction of organisms. The results of these studies are summarized below.

Fresh adult human serum was shown to exert marked bactericidal and lytic action against a strain of *Vibrio comma*. Heated adult serum and fresh infant serum of normal complement titer did not exhibit these properties. However, a combination of these two sera in the proper proportions was almost as bactericidal as fresh adult serum. Fresh infant serum could also be rendered bactericidal by the addition of inactivated specific rabbit antiserum which in itself was likewise nonbactericidal. By inference, then, adult serum must be bactericidal by virtue of its complement and naturally occurring antibody. Also, by measuring the bactericidal effects of fresh infant serum and specific antiserum combined in varying proportions, the degree of bactericidal action was found to be dependent on the amount of complement as well as on the amount of antibody.

In the same study<sup>12</sup> it was further observed that specific inactivation of any one of the four components of human complement destroyed the bactericidal powers of the treated adult serum. Combination of specifically inactivated complements resulted in the restoration of the bactericidal properties almost completely. All four complement components are therefore necessary for bactericidal and bacteriolytic effects. It was also noted that the bactericidal power of normal adult serum could be fortified by the addition of an excess of any of the specifically inactivated complements, the siC' lacking C'1 but containing C'2 and some C'3 and C'4 (Fraction 1PS) being particularly effective. The fortification of the bactericidal action of complement by the addition of C'2 and C'3 is supportive evidence that these two are the components of minimum titer in human complement.

Hemolytic tests run in parallel with the bactericidal experiments indicated a qualitative relationship between these two phenomena.

#### THE FIXATION OF HUMAN COMPLEMENT BY BACTERIA

Aside from the accepted fact that a suspension of bacteria can fix human complement in vitro, little was known of the nature of this fixation. On the basis of the newer knowledge of the structure of complement, a study was made of the nature of complement fixation by six species of bacteria including both coccid and rod forms.<sup>17</sup>

Fixations were performed with unsensitized bacteria and with the same organisms sensitized by specific rabbit antisera. The effects on the complement were determined by the method of specific reactivation after fixation and separation of the organisms. From this study the following conclusions were drawn.

If the quantity of bacteria employed was sufficiently large, or the incubation period sufficiently prolonged, there was a complete loss of hemolytic activity of the complement. Since complete removal or inactivation of at least one component is necessary for hemolytic inactivation, at least one component must then have been entirely fixed to account for this effect. In agreement with this, at the initial point of removal of over-all hemolytic activity, C'2 or C'3 was found to have been removed completely, and there was a partial decrease in the ac-

tivities of the other components. One exception was noted in the case of *Streptococcus viridans*, in which fixation of 50 per cent of C'2 and C'3 resulted in the complete inactivation of the complement. It is worthy of note that C'2 and C'3 are also the components of minimum titer in human complement.

Further, except for a slight increase in the rate of fixation, no other difference was observed between the effect of sensitized and unsensitized bacteria on the complement. As was pointed out, the presence of natural or group-related antibodies in the sera would tend to obscure expected differences in the nature of fixation in the presence or absence of antibody.

Although definite differences existed in the manner of fixation by various organisms, it was not possible to correlate the mechanisms with any characters of the bacteria themselves. Likewise, definite conclusions regarding the specificity of the fixation cannot be made at this time, although the differences in fixation exhibited by various species of organisms would indicate some degree of specificity. Moreover, one species of organisms, *Neisseria intracellularis*, when employed without antiserum, fixed only a slight amount of C'2 and no other component even after many hours of incubation. Sensitized organisms of this strain fixed all of C'2 after several hours, and a portion of C'4 could be removed on prolonged fixation beyond this time. This particular strain of *Neisseria intracellularis* was resistant to the bactericidal action of several different fresh normal adult sera, which indicated a lack of specific antibody in the sera.

The assay of complement remaining after bacterial fixation revealed which components were fixed to the organisms, but it did not disclose the fate or function of the fixed components. However, in experiments with *Vibrio comma*, it was found that when those components not fixed were added in sufficient amount to the washed treated organisms, bactericidal action resulted. Thus components removed from complement by bacteria were not destroyed but retained their property of participating in subsequent complement reactions. It was further noted that the hemolytic functions of the complement components remaining in the sera after fixation by bacteria closely paralleled the bactericidal functions of the fixed components.

We are convinced that future studies on the mechanism of fixation must take into account the effective concentrations of the individual complement components as suggested in another paper.<sup>22</sup>

In a study of the quantitative aspects of fixation of human complement to specific immune precipitates of egg albumin, the specific carbohydrate of the Type III pneumococcus (S III), and S II, Heidelberger and Mayer<sup>20</sup> concluded that from 0.03 to 0.05 mg. of C' nitrogen per milliliter of human serum adds to these precipitates.

#### THE OPSONIC ACTION OF HUMAN COMPLEMENT

In 1941, before the human complement system had been adequately investigated, Ecker, Pillemer, and Kuehn<sup>28</sup> studied the opsonic action of normal human serum on a strain of *Staphylococcus aureus*. No relationship was found to exist between the hemolytic titer of human serum and its antistaphylococcic opsonic powers. Heat inactivation readily and markedly reduced the normal human serum opsonins. Ammonia, used in what is now known to be approximately

optimum amounts for the inactivation of human C'4, caused reduction of opsonic power; adsorption of the serum with zymine (a yeast preparation) did not change the opsonic capacity. Separation of human serum by carbon dioxide-saturated water into a precipitate (corresponding roughly to Fraction 1PP above) and a supernate (roughly Fraction 1PS) resulted in a division of the opsonic power between the two fractions, the major portion residing in the supernate.

In the past, Gordon and Thompson,<sup>39</sup> Elvidge,<sup>40</sup> and Welch, Brewer, and Hunter<sup>41</sup> have presented evidence to show the mutual independence of opsonin and complement. Ward and Enders<sup>42</sup> summarized the matter by observing that human complement does not produce an absolute increase in the promotion of phagocytosis by the serum, but, acting probably as a biocatalyst, accelerates the rate of phagocytosis.

While it is already obvious that the *total* complement complex is not required for the acceleration of opsonification, experiments now in progress with purified components will determine which components are of importance in the opsonic reaction.

#### THE VARIATIONS OF COMPLEMENT IN INFECTIOUS DISEASE

In 1902 Wassermann<sup>43</sup> proposed the view that the serum complement concentration could be employed as an index of general resistance. Cadham<sup>44</sup> thought that a high complement titer was beneficial in immunity. That low general immunity may be coincident with low complement titer was shown in the case of the complement-deficient guinea pigs bred by Moore<sup>45</sup> in 1919. The sera of these animals were deficient in C'3<sup>46, 47</sup> and perhaps C'4, and the animals themselves were highly susceptible to natural infections and to the injection of pathogenic bacteria.

In general, the complement of an individual is very close to, and constant with, the titer found to be average for the species. This statement applies only to the complement of healthy individuals, however, for the literature lists many variations in disease. In 1912 Dick<sup>48</sup> found that in pneumonia the complement is low before crisis and high the second to third day following crisis. Recently, Rutstein and Walker<sup>49</sup> reported that complement diminishes during the course of pneumonia and particularly following the administration of an antipneumococcal serum. These authors also reported a drop in over-all complement titer in six of eleven cases of serum disease. Thomas and Dingle<sup>50</sup> observed that the intravenous injection of rabbits with a concentrated rabbit antimeningococcal serum resulted in the disappearance of hemolytic complement for as long as twenty-four hours. Interesting clinical responses have also been reported in cases in which fresh serum, containing complement, was used together with the specific sera in meningococcal and influenzal infections.<sup>51-53</sup>

In 1929 da Costa Cruz<sup>54</sup> reported a study of 103 cases of yellow fever, in 94 per cent of which he had observed a markedly diminished complement which rapidly improved in titer during convalescence. It should, however, be emphasized that the liver is diseased in yellow fever, and the low complement may in some measure be related to the interference with production of the protein components of complement.

The literature on complement in tuberculosis is contradictory, as is that on syphilis. In both trypanosomiasis and malaria decreased complement titers have been noted.<sup>1, 2</sup>

With the view of resolving, if possible, the many contradictory reports on variations of complement in infectious disease and determining the mechanisms underlying these variations, we have undertaken a long range and serial study of a variety of cases. In this review only a few tentative conclusions, obtained in a study of approximately 300 cases, are reported as follows:

1. While over-all complement titer often changes in the course of infectious disease, the extent and direction of the changes are by no means always similar in different diseases, nor even in individual instances of the same disease.

2. In fourteen cases the over-all complement fell to zero or near zero, usually coinciding with a serious phase of the patient's condition. With improvement of this condition the complement titer would tend to return to the normal value. However, of these fourteen patients, approximately one-third died, whereas the fatalities of all cases amounted to less than 5 per cent.

3. In almost all of those patients showing a decrease in over-all complement titer there was a striking decrease or disappearance of the *fourth component* (C'4) and a decrease in the second component (C'2). For instance, all of those showing zero complement had no C'4, a fact made more striking if it is recalled that this component is a component of maximum titer in normal complement. C'1 varied only slightly, while C'3 did not vary significantly. When diminished titers improved, there was a concomitant improvement in C'4 and C'2 titers.

4. There appears to be some prognostic value in the complement titration in those patients in whom complement titer decreases, and in particular in following C'4 activity. However, too few cases have been studied to permit this statement to be made with certainty.

5. In numerous cases of scarlet fever, measles, and erysipelas, the patients showed *increased* complement titers, all four components apparently participating in this change.

A detailed analysis of these cases will be published elsewhere, and the relationships of complement titer to serum protein concentration, white blood counts, and temperature will be considered. Future work will be concerned with the relationship of *in vivo* complement fixation, if this is shown to occur, to the lowering of complement titer and with a study of complement variation in the sera of rabbits with experimentally produced infections. Eventually, treatment of infected rabbits will be attempted with purified or concentrated complement preparations.

#### SUMMARY

A constitutional basis for human complement, similar to, though not identical with, that previously established for guinea pig complement, has been presented. Human complement is composed of at least four components, each of which is described by a method of specific inactivation or separation. Three of these have been prepared from human serum by various fractionation procedures, and only the third component (C'3) remains to be purified adequately. It is established that the second and third components (C'2 and C'3) are the

components of lowest titer in human complement and thus determine the over-all titer of the serum.

All four components are required for the immune hemolytic and bactericidal reactions, but the exact role of the components in the acceleration of opsonification is as yet unknown. Six species of bacteria, including coccal and bacillary forms, have been shown to inactivate human complement, principally by the fixation of the second or third components. The fixed complement components retain their capacity for participation in subsequent immunologic reactions such as bactericidal action.

Complement varies in infectious disease, and in those patients in whom it drops in titer to zero or near zero, the prognosis is grave. When the complement disappears in these, it is by virtue principally of the inactivation, fixation, or inhibition of the fourth component (C'4) and secondarily of the second component (C'2).

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# LABORATORY METHODS

## TUBULAR RESORPTION TEST

WITH RESULTS OBTAINED IN NINETEEN CASES OF ACUTE NEPHRITIS

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**H**YPOPHYSIS posterior lobe extracts have been used repeatedly for estimating renal function<sup>1-14</sup>. The various methods consist essentially in determining the specific gravity of urine following the administration of a quantity of extract; the conclusion has been reached that when the kidney loses its capacity to concentrate urine, the antidiuretic hormone also loses its effect. Since 1940<sup>15</sup> we have been using pitressin to test the functional capacity of the kidney, but, instead of taking into account urine concentration, we have made direct measurements of the antidiuretic effect obtained after standardized administration of water and pitressin.

This paper gives a simplification of the technique originally used and the results obtained in nineteen patients with acute nephritis.

*Procedure.*—The method is as follows:

1. The patient is kept fasting and lying in bed
2. One liter of water in ten minutes is given.
3. Five units of pitressin are injected intramuscularly.
4. A catheter is introduced into the bladder and two urine specimens collected at thirty and ninety minutes, respectively.
5. The first specimen is discarded; the second is measured and the figure obtained is the result of the test.

The maximum antidiuretic effect is obtained between thirty and ninety minutes after the pitressin injection.

*Results in Normal Subjects.*—This tubular resorption test was carried out in forty normal male individuals between the ages of 20 and 25 years.<sup>15</sup> The results obtained varied between 16 and 76 c.c. of urine, with an average of 36.2 c.c. We have never seen normal subjects with values higher than 80 c.c., and, therefore, we consider as abnormal any values above this figure.

The test is well tolerated; there is obvious vasoconstriction of the skin and a rise of from 20 to 30 mm. in blood pressure, not lasting more than 15 to 20 minutes after the pitressin injection.

*Results in Acute Nephritis.*—The test was carried out in nineteen cases of acute nephritis occurring in men between the ages of 19 to 21 years. These patients presented the typical picture of acute nephritis, the important symptoms and evolution of which are detailed in Table I. They were admitted to

Hospital Militar Central.  
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TABLE I

## TUBULAR RESORPTION TEST IN ACUTE NEPHRITIS

CASE	DAY FROM THE ONSET OF DISEASE	T.R.T.	U.C.	BLOOD PRES- SURE	HEMA- TURIA	ALBU- MINURIA	URI- NARY CASTS	EDEMA	BLOOD UREA (MG. %)	SED. RATE
1	9	68	Mx 75	150/ 90	+	+	0	+	26	112
	35	62		110/ 64	0	0	0	0		18
	42	-								44
	49	112		118/ 72	0	0	0	0		
	85	87		125/ 75	0	0	0	0		2
2	9	210	Mx 100	168/ 70	++	+	+	+	54	18
	16	154	Mx 66	140/ 70	+	+	0	0	45	18
	30	64		130/ 80	+	0	0	0		15
	44	67		126/ 80	0	0	0	0		5
	58	84		130/ 78	0	0	0	0		
	77	47		130/ 85	+	+	0	0		5
3	15	79	Mx 65	158/ 85	+	0.2 Gm. %	+	+	63	75
	24	111	Mx 125	124/ 62	+	+	+	0	35	20
	37	40		110/ 70	0	0	0	0	48	5
4	8	71	Mx 64	160/100	++			+	50	10
	16	134		150/ 90	+	0	0	+	40	10
	26	70		128/ 70	0	0	0	0	40	10
	45	129		136/ 68	+	0	0	0		2
	53	84		124/ 70				0		5
5	11	158	Mx 93	190/110	+	0.25 Gm. %	+	+	50	10
	20	93		130/ 90	0	0	0	0	30	37
	35	82		120/ 70	0	0	0	0		27
	53	65		120/ 70	0	0	0	0		2
6	27	107	Mx 100	136/ 80	+	+	+	0	64	32
	38	79	Mx 60	130/ 90	+	+	+	0	34	3
	48	60		130/ 80	+	+	+	0		9
7	25	220	Mx 50	170/110	+	0.10 Gm. %	+	+	60	50
	36	165		158/108	0	+	0	0	40	20
	52	86		138/ 96	+	+	+	0	44	20
	66	64		130/ 80				0		10
8	29	60	Mx 71	110/ 70	+	0	0	+	55	10
	44	118		108/ 60	+	+	+	0		2
	55	154		110/ 70	0	0	0	0	35	2
	72	79		126/ 74	0	0	0	0		
9	5	-	Mx 90	178/120	+	+	+	+	64	20
	19	130		150/120	0	0	0	0	27	5
	31	66		145/110	0	0	0	0		5
	42	65		132/ 92	0	0	0	0		5
	62	56		124/ 86	0	0	0	0		2
10	5	-	Mx 78	240/140	+	+	+	+		21
	17	35	Mx 89	170/105	+	+	0	+	56	8
	32	46		166/ 65	0	0	0	0	20	4
	48	30		144/ 75	0	+	0	0		
11	20	-	St 32	210/120	+	0.20 Gm. %	+	+	45	42
	33	75	Mx 33	150/ 90	+	0.10 Gm. %	0	0	70	46
	47	145		122/ 82	+	+	0	0		72
	68	66	Mx 66	142/ 84	+	+	0	0		35
12	3	-	Mx 100	190/140	+	0.20 Gm. %	0	0	56	
	16	78		150/110	+	0.02 Gm. %	0	0		
	34	81		155/110	0	+	0	0	55	
	48	71		156/108	0	+	0	0	55	
	91	74		160/110	+	+	+	0		
13	19	-		145/ 85	+	+	0	0	42	20
	29	105		136/ 70	+	+	0	0		25
	43	70		140/ 90	0	0	0	0		5
	57	87		150/ 86	++	+	+	0		10
	80	92		142/ 90	+	0.01 Gm. %	0	0	49	2
	100	79		134/ 90	+	+	+	0		2
	110*	-		130/ 84	+++	+	+	0		25
	122	86		133/ 82	+	+	0	0		10

\*Tonsillectomy.

†Dental extraction; apical granuloma.

TABLE I—CONT'D

CASE	DAY FROM ONSET OF DISEASE	T.R.T.	U.C.	BLOOD PRES- SURE	HEMA- TURIA	ALBU- MINURIA	URI- NARY CASTS	EDEMA	BLOOD UREA (MG. %)	SED. RATE
14	22	138		160/110	+	0.05 Gm. %	0	+	55	25
	36	167	Mx 52	134/ 80	+	+	+	+	64	40
	53	180	Mx 70	124/ 80	+	+	+	0		90
	66	145		124/ 75	+	+	0	0	50	90
	82	127		130/ 80	+	+	0	0	50	40
	102	107	Mx 72	128/ 82	+	+	0	0		30
	113	88		132/ 85	+	+	+	0		10
	129	72	Mx 129	125/ 85	+	+	+	0		10
15	13	142	Mx 63	170/ 95	++	0.15 Gm. %	+	+	75	54
	27	116	Mx 47	130/ 80	++	0.20 Gm. %	0	0	30	62
	41	159		130/ 70	+	+	0	0		49
	67	99		128/ 60	+	+	+		40	23
	82	113	Mx 81	128/ 60				0		20
	91	97		118/ 60	+	0	0	0	45	16
	109	78	Mx 152	126/ 62						5
	123	84		120/ 64						2
16	11	-	St 29	160/130	+	0.05 Gm. %	+	+	145	40
	34	85	Mx 85	130/100	+	+	0	0	17	20
	41	40		120/ 80				0	22	20
	54	79		120/ 78	0	0	0	0		6
	93	63		120/ 80	0	0	0	0		3
17	17	-		150/ 60	+	0.05 Gm. %	0	0	30	70
	34	90		130/ 70	+	0	+	0	36	38
	43	94		132/ 60	+	0	0	0		30
	56	95		120/ 75	0	0	0	0		19
	85	61	Mx 100	122/ 70	0	0	0	0		8
	108	49		120/ 70	0	0	0	0		
18	9	120	Mx 93	180/100	++	0.025 Gm. %	+	+	60	85
	16	75	St. 61	130/100	0	+	0	0	35	65
	30	79		132/ 80	0	0	0	0		10
	44	62		140/ 95	0	0	0	0		2
	58	59		132/ 88	0	0	0	0		
	60†	-								
	65	34		134/ 82	+	+	0	0		5
	101*	-		120/ 80	+	+	+	0		42
	106	45		120/ 80	+	+	+	0		25
19	4	-	St 93	155/ 70	+	0.025 Gm. %	+	+	150	70
	14	134	Mx 42	142/ 76	++	0.025 Gm. %	+	0	90	90
	50	56	Mx 170	142/ 66	0	0	0	0	42	85
	70*	-		130/ 60	++	0	0	0		
	105	50		120/ 62	0	0	0	0		26

the hospital early in the course of the disease and remained for periods which varied between 45 and 135 days. The treatment consisted of rest, diet, and general care only.

The test was made at an early stage and repeated periodically throughout the patient's stay in the hospital. The following determinations were made simultaneously: urine analysis, blood urea, sedimentation rate, blood pressure, and, occasionally, urea clearance test. An average of five tests was performed on each patient, the results of which are detailed in Table I. They were as well tolerated as in normal subjects.

Only one patient gave a normal value; abnormal figures, for example, higher than 80 c.e., were obtained in eighteen patients. The antidiuretic action of pitressin was decreased in all patients and had even disappeared completely in some of them; that is, after the ingestion of a liter of water the diuresis was the same with or without pitressin.<sup>16</sup> The results obtained in the first stages of

the disease were, in general, the most abnormal, gradually decreasing to normal values toward the later stages.

In fourteen of the eighteen patients it was possible to repeat the test until the normal level had been reached. This occurred between 30 and 135 days after the appearance of the disease. In ten of these patients all other symptoms of the disease had disappeared by that time, while in four there were still signs of hematuria, albuminuria, and urinary casts.

In nine of the eighteen patients, the last abnormal test, when recorded, was the only remaining sign of the disease; in six of these it eventually returned to normal. On the other hand, in the remaining nine the last abnormal test was still accompanied by hematuria, albuminuria, urinary casts, or increased sedimentation rate.

In the patients in whom it was possible to carry out urea clearance tests, the results obtained, with very few exceptions, returned to normal much before the tubular resorption test. The blood pressure always went back to normal much before the tubular resorption test.

#### DISCUSSION

Pitressin acts directly on the renal tubules, causing an increase in water resorption and consequently a decrease in diuresis.<sup>17, 18</sup> This antidiuretic effect is decreased or disappears completely in dogs intoxicated with uranium salts, as demonstrated by Molitor and Pick<sup>19</sup> and Hayman, Shumway, Dumke, and Miller.<sup>20</sup> As is well known, the uranium salts selectively affect the renal tubules. It is most probable that when the renal tubules are damaged in man, whatever the cause, the antidiuretic effect is also diminished or even disappears and that the extent of the diminution is proportional to the extent of the tubular lesions.

As water resorption can undoubtedly be considered one of the principal functions of renal tubules, we find that in estimating the loss of that function, a direct measurement of urine volume seems more appropriate than does the indirect method of measuring urine concentration. Moreover, we consider that determining urine volume instead of urine concentration has the following advantages: (1) urine volume is independent of salt and protein content of the diet taken during the previous days; (2) it eliminates the necessity of giving a test meal; (3) it is less affected by the presence of edema. From a practical standpoint the whole test lasts only one and one-half hours and is well tolerated even in the early stages of nephritis.

Results obtained in acute nephritis lead us to presume that tubular function is affected at a very early stage<sup>16, 21</sup>; moreover, the test tends to normalize itself very late, generally after all other signs of the disease have disappeared, indicating that in many cases tubular function is the last to return to normal. As only one of the nineteen patients gave normal values for the tubular resorption test in spite of the presence of the other symptoms, it might be that the disease in this case was of a different kind and that consequently there might exist two types of acute nephritis.

#### SUMMARY

A tubular resorption test using the antidiuretic hormone of the posterior pituitary has been proposed for estimating renal tubular function. The tech-

nique consists of the following procedures: after the ingestion of 1 liter of water and the injection of 5 units of pitressin, urine is collected during the interval between thirty and ninety minutes after the water intake. In normal subjects the diuresis during that period was from 16 to 76 c.c. (average, 36.2); a diuresis of more than 80 c.c. is considered abnormal.

Of nineteen patients with acute nephritis, eighteen gave abnormal values during the course of the disease with, in general, a decrease toward normal values in the later stages. In most of the patients the tubular resorption test was one of the last signs to return to normal.

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# A SYNTHETIC MEDIUM FOR DETERMINING CITRATE-UTILIZING, HYDROGEN SULFIDE-FORMING BACTERIA

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KOSER,<sup>1</sup> working with the salts of certain organic acids, demonstrated that the genus *Aerobacter* can be distinguished from *Escherichia coli* through the ability of the former to utilize citric acid as a sole source of carbon, whereas *Esch. coli* cannot. The ability to utilize citric acid as a sole source of carbon is shared, also, by *Escherichia freundii* and other intermediates of the coli-aerogenes groups as well as by most species of the *Salmonella* and by many of the "paracolon" group of organisms.

In 1926, Simmons<sup>2</sup> adapted Koser's findings into his citrate agar medium.

*Esch. freundii*, most of the genus *Salmonella*, and the "true" paracolons, which cannot be distinguished from the genus *Aerobacter* on either the Koser or the Simmons media, may be separated from it by their ability to form hydrogen sulfide on peptone media.

It occurred to me that possibly the value of these citrate media might be enhanced if a suitable hydrogen sulfide indicator could be incorporated into one or both of them in order to differentiate further those organisms capable of utilizing citric acid as a sole source of carbon. If this were found to be possible, it might eliminate the necessity of using two different media for this purpose.

Since Koser<sup>1</sup> noted that "identical results could be secured with either potassium, ammonium, or sodium citrate," there was the possibility that ferric ammonium citrate might be used as the source of both citrate and iron. The citric acid content of sodium citrate and ferric ammonium citrate is approximately the same.

The use of iron as an indicator of hydrogen sulfide formation, of course, is not new. Wilson,<sup>3</sup> Schunk,<sup>4</sup> Beckwith and Moser,<sup>5</sup> Levine, Vaughn, Epstein, and Anderson,<sup>6</sup> Sulkin and Willett,<sup>7</sup> ZoBell and Feltham,<sup>8</sup> and others have reported on the use of ferric chloride, ferric citrate, and ferrous sulfate, while ferric ammonium citrate has been used by the Difco Laboratories<sup>9</sup> as a hydrogen sulfide indicator in a peptone-carbohydrate medium for several years.

Substitution of ferric ammonium citrate for sodium citrate in the usual citrate media, however, lowered the pH sufficiently to cause hydrolysis of the agar upon autoclaving. Therefore, the composition of the medium was altered and the constituents so adjusted that the reaction was slightly above pH 7. This not only produced a medium stable to autoclaving, but also brought it into the sensitive range of the iron indicator system. Sulkin and Willett<sup>7</sup> and Hunter and Crecelius<sup>10</sup> have shown that ferric ammonium citrate reacts best as

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an indicator of hydrogen sulfide in an alkaline (pH 7.5) menstruum. As this synthetic medium, after inoculation, progressively becomes more alkaline with growth of the organism, the ferric ammonium citrate functions efficiently as an indicator well within its sensitive range.

The emended formula is as follows: magnesium sulfate, 0.02 per cent; sodium ammonium phosphate, 0.15 per cent; sodium chloride, 0.5 per cent; dipotassium phosphate, 0.1 per cent; ferric ammonium citrate (green), 0.3 per cent; sodium thiosulfate, 0.3 per cent; sodium sulfite, 0.04 per cent; agar, 2.0 per cent. The final reaction is slightly above pH 7 with no adjustment required. The medium should be stirred before and again after heating.

*Agar Slants.*—The medium is tubed and slanted to provide a generous butt. The organism (free of substrate) is stabbed through the medium to the bottom of the tube and then streaked over the surface of the slant. At 37° C. intense blackening of the medium first appears along the stab or in the water of condensation, or both, in about fifteen or sixteen hours and continues to spread until the entire butt is jet black. The surface growth on the slant is yellow against the whitish agar. This growth becomes orange as incubation continues and furnishes direct evidence of growth. Heavy inocula which refuse to grow on this medium do not assume this deep yellow color.

Contrary to the findings of Vaughn and Levine,<sup>11</sup> the concentration of agar seems to increase sensitivity of the medium to hydrogen sulfide. However, there is little or no increase in sensitivity above 1.5 per cent of agar.

In several respects, evidence of hydrogen sulfide formation in synthetic medium differs from that in conventional peptone media, as will be noted from Tables I and II. It is obvious, of course, that there will be no blackening of the medium if the organism itself cannot utilize citric acid as a sole source of carbon. This is illustrated by two of the hydrogen sulfide-forming members of the genus *Salmonella*, *Salmonella choleraesuis* (variety from Kunzendorf) and *Salmonella pullorum*, neither of which utilize citric acid as a sole source of carbon. Inoculation of *S. pullorum* into synthetic medium, to which dextrose had been added as a supplementary source of carbon, was unsuccessful. The organism still was unable either to initiate visible growth or to form hydrogen sulfide during an incubation period of sixty days.

Quite unexpected was the behavior of *Salmonella* sp. (variety from Newcastle). According to Bergey<sup>12</sup> and others, this organism does not produce hydrogen sulfide. Moreover, this particular culture has been tested a number of times over a period of three years on peptone-ferric citrate agar, on peptone-ferrous sulfate agar (Kligler's iron agar) and on peptone-ferric ammonium citrate agar without noting a single instance of hydrogen sulfide formation even after prolonged incubation.

It will be noted in Table I that this Newcastle strain produces a very strong 1 plus blackening of the agar along the stab in this synthetic medium. It is quite conspicuous when the stab is made between the glass and the agar. Control tubes of Kligler's iron agar inoculated simultaneously showed no trace of blackening. The reason for this may only be surmised at present. It may

TABLE I  
SALMONELLA AND PARACOLON GROUPS

ORGANISM*	SYNTHETIC MEDIUM		KLIGLER'S IRON AGAR†
	GROWTH	H <sub>2</sub> S	H <sub>2</sub> S
<i>S. sp.</i> (variety from Newcastle) (1)†	+	+	-
<i>S. pullorum</i> (5)	-	-	+++
<i>S. choleraesuis</i> (1)	-	-	-
<i>S. choleraesuis</i> (variety from Kunzendorf) (3)	-	-	++++
<i>S. typhimurium</i> (2)	+	++++	++++
<i>S. sp.</i> (Derby type) (2)	+	++++	++++
<i>S. sp.</i> (Bareilly type) (2)	+	++++	++++
<i>S. hirschfeldii</i> (1)	+	++++	++++
<i>S. enteritidis</i> (Gaertner) (1)	+	++++	++++
<i>S. sp.</i> (Panama type) (1)	+	++++	++++
<i>S. anatis</i> (Rettger and Scoville) (1)	+	++++	++++
<i>S. sp.</i> (Oranienburg type) (1)	+	++++	++++
<i>S. sp.</i> (Newport type) (2)	+	++++	++++
<i>S. sp.</i> (Senftenberg type) (1)	+	++++	++++
<i>S. sp.</i> (Give type) (2)	+	++++	++++
<i>S. sp.</i> (Montevideo type) (2)	+	++++	++++
<i>S. sp.</i> (Rubislaw type) (1)	+	++++	++++
<i>S. sp.</i> (Worthington type) (1)	+	++++	++++
<i>S. sp.</i> (Lexington type) (1)	+	++++	++++
<i>S. sp.</i> (Kentucky type) (1)	+	++++	++++
<i>S. sp.</i> (Bredeney type) (1)	+	++++	++++
<i>S. sp.</i> (Newington type) (1)	+	++++	++++
<i>S. sp.</i> (California type) (1)	+	++++	++++
<i>S. sp.</i> (Meleagridis type) (1)	+	++++	++++
<i>S. sp.</i> (Berta type) (1)	+	++++	++++
<i>S. sp.</i> (St. Paul type) (2)	+	++++	++++
<i>S. sp.</i> (Illinois type) (1)	+	++++	++++
"True" paracolon (6)	+	-	++++
"Pseudo" paracolon (3)	+	-	-
"Pseudo" paracolon (1)	-	-	-

\*Organisms were typed and their identity confirmed by Dr. P. R. Edwards of Kentucky Agricultural Experiment Station.

†Numbers in parentheses represent number of different strains used.

‡Difco Laboratories Inc., Detroit, Mich.

TABLE II  
ESCHERICHIA AND AEROBACTER GENERA

ORGANISM	SYNTHETIC MEDIUM		KLIGLER'S IRON AGAR†
	SURFACE GROWTH	H <sub>2</sub> S	H <sub>2</sub> S
<i>Esch. coli</i> (18)*	-	-	-
<i>Esch. freundii</i> (5)	+	+	+
<i>A. aerogenes</i> (12)	+	-	-
<i>Coli-aerogenes</i> "intermediates" (8)	+	-	-

\*Numbers in parentheses represent number of different strains used.

†Difco Laboratories Inc., Detroit, Mich.

be that peptone media have a tendency to suppress hydrogen sulfide formation by this particular organism. On the other hand, Hunter and Crecelius<sup>10</sup> were able to show that many organisms, including *Esch. coli* and *Aerobacter aerogenes* not normally considered as producing hydrogen sulfide, were able to produce blackening of the agar when bismuth was used as a more sensitive indicator of hydrogen sulfide. The ability of this Newcastle strain to form hydrogen sulfide in twenty-four hours or less in synthetic medium again emphasizes<sup>10, 11, 13</sup> that the ability of an organism to form hydrogen sulfide is a function of the particu-

lar medium used, and that whenever hydrogen sulfide production by an organism is mentioned, it is important that the particular medium used be mentioned also.

All of the other *Salmonella* cultures in our stock collection (except *S. choleraesuis*, *S. choleraesuis* [variety from Kunzendorf], and *S. pullorum*) were able to utilize citric acid and to show dense blackening of the medium, usually by the fifteenth hour. No cultures of *Salmonella schottmülleri* or *Salmonella paratyphi* were available for testing on this medium. However, Simmons<sup>2</sup> reports *S. schottmülleri* (hydrogen sulfide-positive) as citrate positive and *S. paratyphi* (hydrogen sulfide-negative) as citrate negative.

Ten strains of paracolons were available for testing on this medium and on Kligler's iron agar. Six of them correspond to Dr. Edward's definition of the "true" paracolon and had been so classified by him. Two of these were of the Z<sub>4</sub> type. The remaining four were nonhydrogen sulfide-forming, sucrose- and salicin-fermenting strains designated as "pseudo" paracolons. All but one strain of the latter (DL-125) were able to utilize citric acid as a sole source of carbon. None of the "true" paracolons had fermented lactose in 264 hours, while all of the "pseudo" strains formed acid and gas from it after 72 to 192 hours.

All paracolon strains except one (DL-125) grew luxuriantly on the surface slant of the synthetic medium. However, only the "pseudo" strains were able to grow equally well *deep under* the surface. The "true" paracolons apparently have difficulty in utilizing citric acid anaerobically and this was reflected in meager growth along the stab (Table III).

TABLE III

ORGANISM	SURFACE GROWTH	SUBSURFACE GROWTH (DEEP STAB)	BLACKENING OF SYNTHETIC AGAR	BLACKENING OF KLIGLER'S IRON AGAR
Paracolon ("true")	+	Slight	-	++++
Paracolon ("pseudo")	+ or -	Profuse (or -)	-	-
<i>S. choleraesuis</i>	-	None	-	-
<i>S. choleraesuis</i> (Kunzendorf)	-	None	-	++++
<i>S. pullorum</i>	-	None	-	+++
<i>S. Newcastle</i>	+	Profuse	+	-
Other <i>Salmonella</i>	+	Profuse	++++	++++

Although the "true" paracolons are vigorous hydrogen sulfide producers on peptone media, all of them persistently have refused to produce any blackening of the synthetic medium in stab inoculations (see below for possible exception).

*Esch. coli*, of course, is unable to utilize citric acid as a sole source of carbon aerobically. However, there is some evidence that at least some strains apparently are able to grow to a slight degree just beneath the surface. This growth seems to be microaerophilic in nature rather than anaerobic and, in this respect, is similar to the habit of the "true" paracolons.

*Esch. freundii* not only utilizes citric acid, but also forms hydrogen sulfide.

The genus *Aerobacter* grows luxuriantly aerobically and anaerobically but does not blacken the medium either with or without agar. Occasionally, bubbles of gas appear in the agar into which *A. aerogenes* has been inoculated. This may



TABLE I  
SALMONELLA AND PARACOLON GROUPS

ORGANISM*	SYNTHETIC MEDIUM		KLIGLER'S IRON AGAR†
	GROWTH	H <sub>2</sub> S	H <sub>2</sub> S
<i>S. sp.</i> (variety from Newcastle) (1)†	+	+	-
<i>S. pullorum</i> (5)	-	-	+++
<i>S. choleraesuis</i> (1)	-	-	-
<i>S. choleraesuis</i> (variety from Kunzendorf) (3)	-	-	++++
<i>S. typhimurium</i> (2)	+	++++	++++
<i>S. sp.</i> (Derby type) (2)	+	++++	++++
<i>S. sp.</i> (Bareilly type) (2)	+	++++	++++
<i>S. hirschfeldii</i> (1)	+	++++	++++
<i>S. enteritidis</i> (Gaertner) (1)	+	++++	++++
<i>S. sp.</i> (Panama type) (1)	+	++++	++++
<i>S. anatis</i> (Rettger and Scoville) (1)	+	++++	++++
<i>S. sp.</i> (Oranienburg type) (1)	+	++++	++++
<i>S. sp.</i> (Newport type) (2)	+	++++	++++
<i>S. sp.</i> (Senftenberg type) (1)	+	++++	++++
<i>S. sp.</i> (Give type) (2)	+	++++	++++
<i>S. sp.</i> (Montevideo type) (2)	+	++++	++++
<i>S. sp.</i> (Rubislaw type) (1)	+	++++	++++
<i>S. sp.</i> (Worthington type) (1)	+	++++	++++
<i>S. sp.</i> (Lexington type) (1)	+	++++	++++
<i>S. sp.</i> (Kentucky type) (1)	+	++++	++++
<i>S. sp.</i> (Bredeney type) (1)	+	++++	++++
<i>S. sp.</i> (Newington type) (1)	+	++++	++++
<i>S. sp.</i> (California type) (1)	+	++++	++++
<i>S. sp.</i> (Meleagridis type) (1)	+	++++	++++
<i>S. sp.</i> (Berta type) (1)	+	++++	++++
<i>S. sp.</i> (St. Paul type) (2)	+	++++	++++
<i>S. sp.</i> (Illinois type) (1)	+	++++	++++
"True" paracolon (6)	+	-	++++
"Pseudo" paracolon (3)	+	-	-
"Pseudo" paracolon (1)	-	-	-

\*Organisms were typed and their identity confirmed by Dr. P. R. Edwards of Kentucky Agricultural Experiment Station.

†Numbers in parentheses represent number of different strains used.

‡Difco Laboratories Inc., Detroit, Mich.

TABLE II  
ESCHERICHIA AND AEROBACTER GENERA

ORGANISM	SYNTHETIC MEDIUM		KLIGLER'S IRON AGAR†
	SURFACE GROWTH	H <sub>2</sub> S	H <sub>2</sub> S
<i>Esch. coli</i> (18)*	-	-	-
<i>Esch. freundii</i> (5)	+	+	+
<i>A. aerogenes</i> (12)	+	-	-
<i>Coli-aerogenes</i> "intermediates" (8)	+	-	-

\*Numbers in parentheses represent number of different strains used.

†Difco Laboratories Inc., Detroit, Mich.

be that peptone media have a tendency to suppress hydrogen sulfide formation by this particular organism. On the other hand, Hunter and Crecelius<sup>10</sup> were able to show that many organisms, including *Esch. coli* and *Aerobacter aerogenes* not normally considered as producing hydrogen sulfide, were able to produce blackening of the agar when bismuth was used as a more sensitive indicator of hydrogen sulfide. The ability of this Newcastle strain to form hydrogen sulfide in twenty-four hours or less in synthetic medium again emphasizes<sup>10, 11, 13</sup> that the ability of an organism to form hydrogen sulfide is a function of the particu-

**Liquid Medium.**—By omitting agar from the formula, the medium acts like Koser's citrate medium except that in addition to turbidity, there also is the orange reddening of the medium as an indication of growth. Hydrogen sulfide formation may be detected by a blackening of sediment at the bottom of the tube.

#### DISCUSSION

It is believed that this new ferric ammonium citrate medium will serve a useful and valuable purpose in several fields of bacteriology. The value of existing citrate media has been confined, principally, to differentiating *Esch. coli* from *A. aerogenes* and the coli-aerogenes intermediates. This new medium materially extends the usefulness of these media by providing a three-fold separation of (a) *Esch. coli* from (b) *Esch. freundii*, and of both of them from (c) *A. aerogenes* and the citrate-utilizing, nonhydrogen sulfide-forming coli-aerogenes intermediates.

Within the genus *Salmonella*, the usefulness of citrate media, in the past, has been restricted to a few species or varieties, such as *S. paratyphi*, *S. choleraesuis*, and *S. pullorum*, which are unable to utilize citric acid as a sole source of carbon. This new ferric ammonium citrate medium becomes an important differential medium for this genus. On it, for example, *S. sp.* (variety from Newcastle) is shown to produce hydrogen sulfide. It is believed that this is the first time detectable hydrogen sulfide formation may have been reported for this organism. Because of this characteristic, this medium offers a valuable means for differentiating *S. sp.* (variety from Newcastle) from the other *Salmonella*. The appearance of the hydrogen sulfide reaction differs so markedly from that of other *Salmonella* that little or no confusion is likely.

This ferric ammonium citrate synthetic medium used in conjunction with one of the conventional peptone-hydrogen sulfide indicator media should be of material aid, also, in the differentiation of *S. pullorum*, *S. choleraesuis*, *S. choleraesuis* (variety from Kunzendorf), and *S. paratyphi*.

A synthetic medium is described which demonstrates the presence or absence of detectable hydrogen sulfide produced by those bacteria capable of utilizing citric acid as a sole source of carbon.

It may be used in agar slants either as a plating medium or (in liquid form) as an alternative to Koser's citrate medium.

Bacteria either grow or do not grow on the medium, depending on whether they are able to utilize citric acid.

Those bacteria which do grow on it may or may not form hydrogen sulfide.

Intense blackening of the agar occurs within fifteen to twenty-four hours after inoculation.

The ability to form detectable hydrogen sulfide seems to be confined specifically to the *Salmonella* group of organisms and to *Esch. freundii*.

*S. sp.* (variety from Newcastle), previously considered as not forming hydrogen sulfide, consistently shows a 1 plus blackening of the agar within twenty-four hours in this medium. This should prove a valuable means of identifying this organism if this characteristic is found to hold true generally for all Newcastle strains.

The Newcastle strain which produces a 1 plus blackening in test tube slants fails to do so in Petri plate inoculations.

The paracolons, which readily form hydrogen sulfide in peptone media, fail to do so in test tube slants of synthetic medium.

The Z<sub>4</sub> paracolon, which does not produce hydrogen sulfide in test tube slants of synthetic medium, produces black colonies on streak-stab Petri plate inoculations.

Some of the Salmonella group which form hydrogen sulfide on peptone media do not grow on the synthetic medium. These two media used together provide evidence of value in differentiating various members of the Escherichia, Aerobacter, Salmonella, and paracolon groups.

It is believed that this may be the first instance where a synthetic or a non-peptone medium has been used to detect hydrogen sulfide formation by bacteria.

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# THE APPLICATION OF A HOT WIRE AND THERMOCOUPLE FOR RECORDING SURFACE PULSATIONS IN THE HUMAN BODY\*

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A GREAT deal of ingenuity has been demonstrated in the development of methods for recording arterial, venous, and other pulsations from the surface of the human body. The older sphygmographs (Wiggers, 1923<sup>1</sup>) employ a button, spring, or cup applied to the skin over an artery and transmit the movement to a lever system or to a column of air connected to a recording tambour. These have been largely supplanted, where accuracy of registration is desired, by instruments having a higher figure of merit. In one type of transmission instrument, the pulsations are picked up by a button, capsule, cup, or plethysmograph and led to a segment capsule or similar device, the movements of which are optically recorded (Wiggers, 1928;<sup>2</sup> Bolton, Carmichael, and Stürup, 1936;<sup>3</sup> Turner, 1937<sup>4</sup>). Pulses in a finger have also been obtained by transmission plethysmographs in which the movements of a drop of fluid (Johnson, 1932<sup>5</sup>) or of a water meniscus (Goetz, 1934,<sup>6</sup> 1940<sup>7</sup>) are photographed. Records of the pulse have also been made in terms of the change in resistance of a carbon microphone (Waud, 1924;<sup>8</sup> Turner, 1928<sup>9</sup>) or of a crystal microphone (Miller and White, 1941<sup>10</sup>) or by the change in electrical capacitance of a condenser unit (Fenning and Bonar, 1939;<sup>11</sup> Fenning, 1943<sup>12</sup>). The demonstration by Bonsmann (1934),<sup>13</sup> that a photoelectric cell is capable of detecting differences in the blood supply to a region, led to the development of photoelectric pulse recorders for regions such as the fingers, toes, ear, and nasal septum (Hertzman and Speelman, 1937;<sup>14</sup> Hertzman, 1937;<sup>15, 16</sup> Matthes and Hauss, 1938<sup>17</sup>). The change in electrical impedance of a region between electrodes with variations in blood supply has been used as the basis of a pulse-recording method by Mann (1937)<sup>18</sup> and Nyboer (1944).<sup>19</sup> Although not giving any results, Asher and Hopf (1935)<sup>20</sup> have devised an interesting arrangement whereby variations in blood supply to a region, such as the arm, result in an unbalance between the outputs of two vacuum tube oscillators; which unbalance, after rectification and amplification, is translated into the deflections of an oscillograph.

To minimize inertia, Hill (1921)<sup>21, 22</sup> used an air transmission system in which the movements of air resulting from each pulse cause a cooling of a very fine, heated wire, thus resulting in a change in resistance of the wire and unbalancing a Wheatstone bridge, of which the wire is a part. As Hill himself points out, this method is of value in determining certain time relations but it gives no accurate picture of the movements of the artery. Wishing to utilize

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the movement of a small mass of air, as Hill did, but hoping to improve the low frequency response of such a system, we have devised a simple arrangement employing a thermocouple and hot wire. Although this method does not give an exact representation of the pulse, it succeeds, as will be shown, in portraying the main components in their proper time relations.

#### DESCRIPTION OF THE PULSE RECORDER

The unit (Fig. 1) consists of a lucite block with a transverse hole, into which are cemented two cylindrical lucite pieces ( $B_1$ ,  $B_2$ ). On one of these pieces is mounted a copper-constantan thermocouple, the diameter of the copper and constantan wire being 0.001 inch. On the other piece is placed a small helical coil ( $h$ ) wound from 0.003 inch diameter nichrome wire. This coil is mounted so that it is aligned with one edge of a 1 mm. opening ( $Q$ ) drilled longitudinally through the lucite block. The active junction ( $t$ ) of the thermocouple is about 0.5 to 1.0 mm. away from the coil but aligned with the center of the opening ( $Q$ ). The basic idea is that the coil, heated by means of an electric current, results in the formation of a small pocket of warm air in front of the active junction. The pulse then causes a movement of warm air against the active junction, resulting in a signal (E.M.F.) which is first amplified and then recorded oscillographically. The heat capacity of the active junction should be minimized by keeping the mass of the soldered joint as small as possible. The inactive junction is, of course, the junction between the constantan wire and one of the copper lead-off wires ( $t.l.$ ). Its mass, and therefore heat capacity, is made large relative to that of the active junction, so that it will be influenced only by slow changes in temperature. One end of the lucite block is provided with a receiver ( $R$ ) having an internal thread into which can be fitted any one of the adapters which are used in leading off from the pulsating surface. To obtain maximum sensitivity, these adapters should fit accurately so that no leaks exist. We have employed plaster of Paris molds of the surfaces to be fitted. Adapters for the finger, the neck (carotid artery), and the wrist (radial artery) are pictured in Fig. 1.

In actual practice the heating current for the coil is supplied by a 3.0 volt source. This is sufficient to bring the coil, whose resistance is 8 to 10 ohms, to a reddish glow. The relatively small signals (50 to 200 microvolts, records  $T$ ,  $U$ ; Fig. 2) are first amplified by means of a three-stage, high-gain, resistance-capacitance-coupled amplifier, of variable time-constant, and then fed into a cathode ray oscillograph. The maximum time-constant of 2.0 seconds (record  $V$ , Fig. 2) was always employed when accurate registration of the signal was desired. The entire instrumental problem can be greatly simplified if a high sensitivity galvanometer of adequate period, such as one of those employed in geophysical work, is available.

#### RESULTS

1. *Finger Pulses.*—It is our experience that the finger pulse is easily and quickly recorded with this method. For success it is essential to use a snugly fitting adapter in which the dead space is reduced to a minimum. The adapter

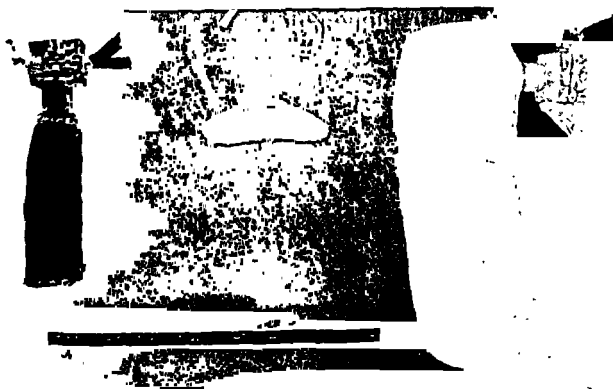
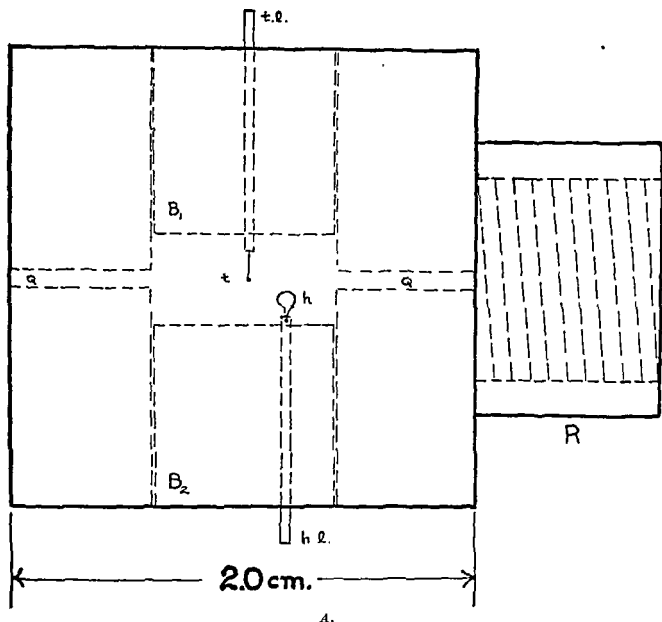


Fig. 1.—A, Diagram of the pulse recorder. The heating coil,  $h$ , is in reality a helix rather than a loop as shown here. Only one of the two thermocouple lead-off wires,  $t.l.$ , and only one of the two heating coil lead-off wires,  $h.l.$ , are illustrated. B, Photographs of the unit with a finger adapter, left, a carotid adapter, center, and a radial adapter, right. Further explanation in text.

can easily be made snug, yet nonconstrictive, by depositing several layers of plaster of Paris bandages over a thin rubber mold placed on the subject's finger. When the plaster has dried, the rubber is removed, leaving the finished adapter. Finger pulses from seven individuals are pictured in records *A* to *H*, Fig. 2. All of these were obtained from healthy adults except for record *II*, which represents the pulse from a 63-year-old patient with hypertension (blood pressure, 160/120) and slight arteriosclerosis.

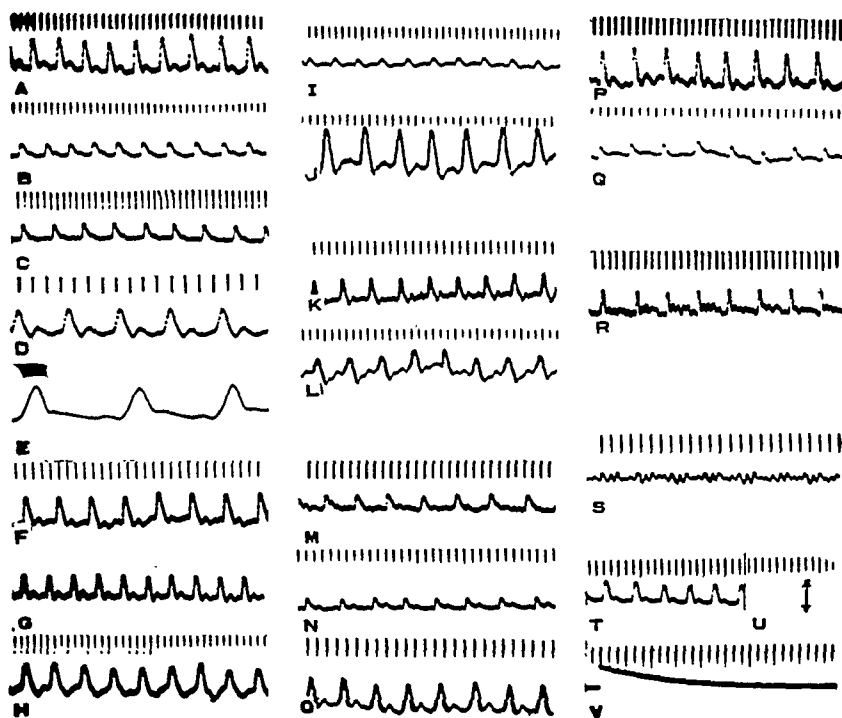


FIG. 2.—Photographs of records taken with the cathode ray oscillograph to show the pulse from various regions of the body. Records *D*, *E*, *I*, *J*, and *T* were all obtained from the same person (*E. G.*); the others were taken from different individuals. Record *T* is a finger pulse to be compared in magnitude with a record, *U*, showing a 100 microvolt, 60-cycle sine wave (arrow shows amplitude). Record *V* shows the response of the amplifier to the "on" of a square wave. The time line in all records indicates 0.2 second.

The wave form of these finger pulses was evaluated by comparing it with the form of the finger pulses obtained from the same individuals but using the photoelectric method. In Fig. 3 are shown tracings made from enlarged projections of the photographic records from three individuals. In this figure the finger pulses obtained with the present method (*B*) are compared with the finger pulses recorded by means of a phototube (*A*). The accuracy of registration of the finger pulse by the photoelectric technique has already been checked by Dillon and Hertzman (1941).<sup>23</sup> It will be seen that the primary wave, the dirotic wave, and the dirotic notch appear in the same time relations in the pulses obtained with both methods. The chief difference between the two pulse forms is that the catacrotic limb, instead of falling slowly as it should, descends

more rapidly in the thermoelectrically obtained pulse. In other words, the distortion is one due to the failure of the thermocouple to record slow changes. This leads to the appearance of the dicrotic wave much lower on the catacrotic limb than it actually should be, but the time relations are accurately reproduced.

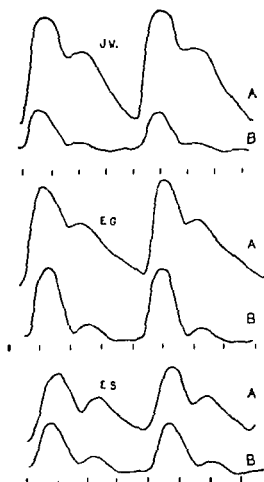


Fig. 3.—The finger pulse from three persons obtained thermoelectrically, *B*, compared with the same pulse recorded with a phototube, *A*. Time line indicates 0.2 second. Explanation in text.

A similar comparison of the pulses obtained by means of the thermoelectric method with the pulses recorded by Dillon and Hertzman (1941),<sup>23</sup> using the photoelectric cell, has led to the same conclusion that appears from a study of Fig. 3. The wave form may be further evaluated by determining the crest time; that is, the time to maximum of the primary wave (Table I). The mean figure for the digital crest time of normal subjects obtained by Dillon and Hertzman (1941),<sup>23</sup> using the photoelectric plethysmograph, is 0.127 second. The difference between the latter figure and that given as the mean in Table I is hardly a significant one.

TABLE I  
FINGER PULSES

SUBJECT	CREST TIME (SEC.)
F. C.	0.114
J. M.	0.127
J. W.	0.125
E. S.	0.131
E. S. S.	0.119
G. S.	0.111
Mean	0.121



It is of some concern to inquire whether differences in details of construction are critical in the operation of the unit and in the results obtained with it. Six units were made according to the general diagram of Fig. 1. In these there are minor variations in the size of the active junction, the resistance of the active junction (3 to 5 ohms), the position of the active junction with respect to the heating coil, and the position of the heating coil with respect to the opening ( $Q$ ). Tracings of the same finger pulse obtained with these six units ( $A$  to  $F$ ) and with a phototube ( $G$ ) are presented in Fig. 4. Aside from differences in sensitivity from one unit to another, the wave forms for the different units is quite similar. Thus if reasonable care is employed, unavoidable variations in construction make no difference in the operation of the unit or in the results obtained through its use.

Another pertinent detail is the relation between the degree of heating of the coil and the operation of the unit, especially with regard to its sensitivity. In records  $F$  to  $M$ , Fig. 5, are presented some finger pulses taken with heating coil currents varying from 320 milliamperes (record  $F$ ) to 150 milliamperes (record  $M$ ). There is a diphasic distortion in these records due to the use of one of the shorter amplifier time-constants, but this does not influence the conclusion that for a considerable range of coil current the unit retains a constant

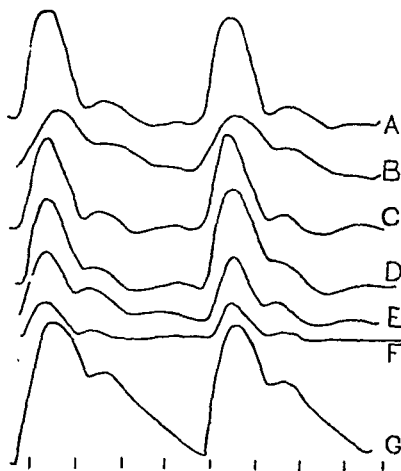
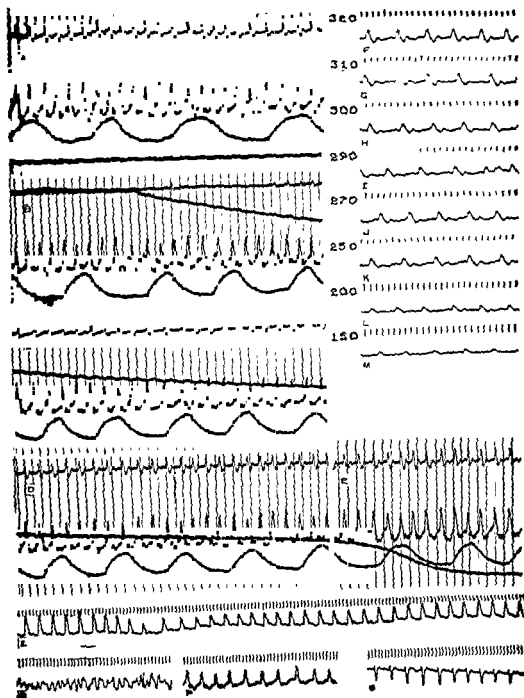


Fig. 4.—The finger pulse obtained from one subject using six different units, records  $A$  to  $F$ , and a phototube,  $G$ . The same amplifier gain was used throughout. Time line indicates 0.2 second. Explanation in text.

sensitivity and yields similar results. It is only when the coil current goes below about 250 milliamperes that a significant loss in sensitivity results. Since the unit is independent of the degree of heating of the coil over a wide range, the possibility of error due to inadvertent variations in the heating current is eliminated. This series of records also illustrates the need for a heating coil, since a thermocouple alone does not possess the same degree of sensitivity as does the combination.

Because of disturbances due to movements of the air, resulting from activity in the hand or fingers, it is essential to know whether the opening ( $Q$ ) must pass

completely through the unit to the outside. To obtain an answer, the finger pulse was first recorded with the opening (*Q*) closed off by a small strip of adhesive (record *O*, Fig. 5). The strip was then removed and the normal finger pulse was obtained (record *P*, Fig. 5). It is obvious that sealing off the opening leads to reflected air waves and interferences which prevent the use of the unit as a pulse recorder. It might be possible to obtain a usable instrument by sealing a cup, the volume of which is large compared to the volume inside the unit, over the end of the lucite block. We have not attempted this, since it is not too difficult for the normal subject to maintain a steady position long enough to



multiple recordings of the finger pulse (upper), carotid (lower), and the cuff pressure (lowest). The two pulse records of too low an amplifier time-constant, were obtained by means of a thermocouple mounted in the air with respiration being recorded with a Cambridge microphone by means of a stretched rubber membrane in the path of a light beam to a barrier being fed into a recording microammeter. Record A shows the cuff pressure starting at 160 mm. and D indicate the gradual fall in pressure. Record N shows the finger pulse. Time line in records

after photocell, the output was taken with the cuff pressure of mercury but slowly to cuff pressure to zero. The time the effect of one deep inspiration F to Q indicates 0.2 second.

record a considerable length of record, but some modification of this type might be necessary in recording the pulse from individuals with an uncontrollable tremor.

2. *Toe Pulse*.—The pulse from the toes is easily obtained with this method if the precautions mentioned in connection with the finger pulse are used in the construction and application of the toe adapter. A toe pulse taken with the same amplification as used in obtaining the finger pulses is shown in record *I*, Fig. 2. The same toe pulse using a higher amplification is given in record *J*, Fig. 2. There is some distortion in this record due to the necessity of having to increase the amplifier screen voltages in order to obtain the higher gain.

3. *The Pulse From Other Regions of the Body*.—The brachial, radial, carotid, and temporal pulses are somewhat more troublesome to obtain properly than are the finger and toe pulses. This is because of the difficulty of getting an adapter that will fit snugly and properly in relation to the pulsating surface and because of movements which produce artifacts and disturbances. The brachial pulse (*K, L*), the radial pulse (*M, N, O*), the carotid pulse (*P, Q*), the temporal pulse (*R*), and the apex beat (*S*) are presented in Fig. 2. For a comparison of the pulse wave form from various regions, tracings made from enlarged projections of the photographs are presented in Fig. 6. Records *A, B*, and *C* were obtained from the same person and show, respectively, the finger, the carotid, and

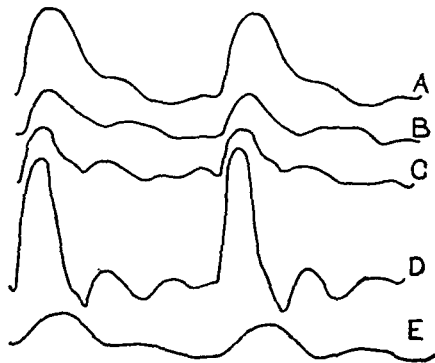


Fig. 6.—The pulse from various regions of the body compared. Explanation in text.

the radial pulses. The brachial pulse (*D*) was taken from a second individual, while record *E* is a toe pulse from still a third person. It appears from these tracings and from the actual records that not only the dicrotic wave but also predicrotic and postdicrotic components are brought out.

A poorly fitted adapter in regions such as the carotid, radial, brachial, or temporal may result in a deflection of reversed polarity. An example of this is indicated in record *Q*, Fig. 5, where a small upward deflection (in the usual direction) is followed by a main swing downward. Whenever this situation has arisen, it has been possible to correct it by either changing the position of the adapter or by replacing it with a better fitting one. Apparently in such cases a leak between the skin surface and the adapter results in the introduction into

the unit of room air which is cooler than the air normally within the unit. As a consequence there occurs, with each pulse, a cooling, rather than a warming, of the active junction.

#### DISCUSSION

It would appear from the introduction to this report that a sufficient number of adequate techniques for recording surface pulsations already exists and that another method is merely superfluous. It is debatable, however, whether a simple, portable, and accurate method capable of registering the pulse from various regions of the body is available. The methods employing segment capsules and optical registration are excellent for research work but are not convenient for use in a portable sphygmograph. Some methods, such as those of Asher and Hopf (1935)<sup>20</sup> and Nyboer (1944),<sup>19</sup> make use of relatively complex electronic circuits which might require repeated attention. The photoelectric method is ideal for recording the pulse from the pinna, the finger, and other regions where a sufficient amount of light is transmitted, but its use in such regions as the wrist and neck presents major difficulties. The methods of Johnson (1932)<sup>5</sup> and Goetz (1934)<sup>6</sup> make use of relatively simple and portable apparatus, but there is always the question of whether enough sensitivity is available for all circumstances. It is possible that by employing a photoelectric cell instead of photographing the movements of the drop or meniscus, an increase in sensitivity could be achieved, but the figure of merit of such a system should

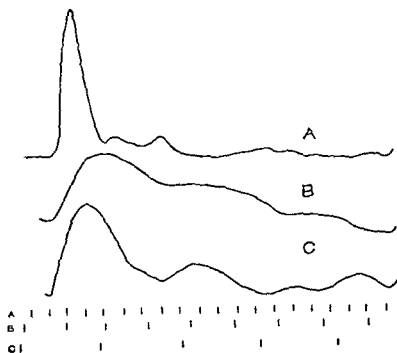


Fig. 7.—The radial pulse taken by three methods. *A* is a hot wire record, *B* is a Frank capsule record, and *C* is a record taken with the method described in this report. Time line for *A* is 0.04 second; for *B*, 0.1 second; and for *C* 0.2 second. Explanation in text.

be under suspicion until adequately checked. The techniques which employ a piezo-electric crystal (Miller and White, 1941<sup>10</sup>) and a capacity pick-up (Fenning and Bonar, 1939<sup>11</sup>) have not been fully tested, but they do offer interesting possibilities. The method of Hill (1921)<sup>21, 22</sup> is intriguing because of its simplicity and because it reduces problems of inertia to a minimum, but the pulse wave form obtained with the hot wire departs considerably from the true wave form. A comparison of the radial pulse taken by (1) the method of

Hill, (2) a Frank capsule, and (3) the thermoelectric method herein described is made in records *A*, *B*, *C*, respectively, of Fig. 7. Record *A* was reproduced from a figure given by Hill (1921),<sup>22</sup> while record *B* was traced from a record in the paper by Bazett and Dreyer (1922).<sup>24</sup> It will be seen that the primary deflection occurs very early in the pulse recorded with the hot wire and that the dicrotic wave is not indicated at all. These facts were recognized by Hill, who did not intend that his method be used in any way except in measuring pulse velocity and time relations in circumstances where knowledge of the true wave form is not necessary.

The present thermoelectric method shares, along with the hot wire technique, the advantage of a system with minimum inertia. It should be emphasized that it, too, does not give a truly exact picture of the pulse, although it does portray the main contours in their proper time relations. The method is extremely simple to use, especially when recording the finger pulse, and it may serve a useful function in problems where the low frequency distortion is of no consequence. Although we have employed a rather complicated amplifier coupled with a cathode ray oscillograph, there is no good reason why a fairly simple and stable amplifier feeding into a sensitive but rugged galvanometer could not be employed, thus making the entire apparatus compact and readily portable.

It might be of value to indicate some possible applications of the thermoelectric technique. Records *A* to *E*, Fig. 5, show the left index finger pulse, the carotid pulse, respiration, and the pressure in a cuff about the left arm. A measurement of the time displacement of the pulse waves in the carotid and finger pulses, a displacement which is clearly evident in the records, plus a measurement of the added distance traveled by the pulse in reaching the finger, should give a figure for the calculation of the pulse velocity. For the photographs in record *A*, the pulse velocity, when the above measurements were made, was 8.2 meters per second. The records (*B*, *C*) showing the appearance of the pulse as the cuff pressure is slowly reduced from 160 mm. of mercury indicates the possible use of this unit in blood pressure determinations. A record of the effect of one deep inspiration (record *N*, Fig. 5) indicates the ability of the unit to detect peripheral circulatory changes. It has been pointed out by a number of investigators (Dillon and Hertzman, 1941<sup>23</sup>) that the digital crest time is increased in such conditions as arteriosclerosis and hypertension. We have had occasion to examine one patient with hypertension; a record of the finger pulse is shown in record *H*, Fig. 2. The mean crest time for this pulse was 0.169 second, whereas the mean value for six normal subjects (Table I) was 0.121 second. This one observation, of course, has no statistical meaning, but it does indicate the need of examining the method further for possible use in cases where a change in configuration of the pulse wave occurs.

#### SUMMARY AND CONCLUSIONS

1. For the registration of the pulse from the surface of the human body a method is described which employs a heating coil and thermocouple. The method makes use of the E.M.F. developed across the two junctions of the

thermocouple as a small mass of warm air impinges upon the active junction with each pulse.

2. Records are shown of the pulse obtained from the fingers, toes, radial artery, brachial artery, carotid artery, temporal artery, and the apex beat. The precautions necessary for getting a successful record with this method, as well as some of the technical details connected with its operation, are pointed out.

3. The method has been evaluated by comparing the finger pulse obtained through its use with records of the finger pulse recorded by means of the photoelectric plethysmograph. It is shown that the primary pulse wave and the secondary waves are portrayed in their correct time relations. The digital crest time agrees well with figures previously published and obtained with the photoelectric technique. The chief limitation of the method results from its poor low frequency response, so that the catacrotic limb descends prematurely, causing the dicrotic notch and dicrotic wave to appear low in the record.

4. A number of possible applications for the technique are suggested, including its use in the measurement of the pulse velocity, its use as a unit in blood pressure determinations, and its possible employment in cases of disturbance in the time relations of the components of the pulse wave.

We wish to express appreciation to Dr. Gordon H. Scott for his help and encouragement throughout the progress of this work. To Mrs. Lois L. Schwartz are due our thanks for the construction of the photoelectric pressure recording unit mentioned in this paper, and to Dr. A. W. Martin of the University of Washington, Seattle, we are indebted for the loan of the oscillograph, the amplifier, and accessory pieces of equipment.

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## REMOVAL OF BACTERIAL PYROGENS FROM PROTEIN HYDROLYSATES

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THE intravenous administration of amino acids has become of increasing importance in medicine within the last five years. Purified amino acids have been used for this purpose in some instances.<sup>1, 2</sup> The cost is high, however, and as a result the amino acids have usually been provided by protein hydrolysates.<sup>3-6</sup> The preparation of enzymatic<sup>7, 8</sup> and acid<sup>9</sup> hydrolysates of protein has been described, with casein the protein of choice. The nutritional quality and utilization of the final hydrolysates have been studied in laboratory animals.<sup>7, 9, 10</sup> An important requirement of such hydrolysates, in common with all substances for intravenous use, is freedom from bacterial pyrogens (fever-producing substances).<sup>11</sup> A method for removing pyrogen from crystalloidal solutions has been described,<sup>12</sup> but there have been no reports on the removal of pyrogen from protein hydrolysates. Procedures for determining the concentration of pyrogen and its reduction and eventual elimination from protein hydrolysates are reported herein.

### EXPERIMENTAL

*Determination of the Concentration of Pyrogen.*—A standard test for the presence of bacterial pyrogen is now available;<sup>15, 16</sup> however, the range of concentration of pyrogen over which a proportional temperature rise is obtained in the rabbit is too short to be utilized for quantitative purposes. Typical data for the temperature rises obtained with various amounts of purified pyrogen are shown in Table I. Similar data have been published by others.<sup>13</sup> Recently the leucocytosis accompanying the injection of pyrogen was reported<sup>14</sup> to be approximately proportional to the amount of pyrogen. However, the data show that the proportionality is no better than that indicated by the temperature rise.

The concentration of pyrogen was determined from the minimal reactive dilution.<sup>18</sup> This phase of the study was facilitated by a preparation of a purified pyrogen, helpful both for determining the amount of pyrogen and the capacity of filters to remove pyrogen. From the data in Table I and other comparable experiments it was concluded that approximately 0.01  $\mu\text{g.}$  of pyrogen will give a perceptible temperature rise in rabbits. In testing for the pyrogen content, protein hydrolysates are diluted until an equivalent perceptible temperature rise is obtained on injection into rabbits. The pyrogen content can readily be calculated from the dilution of the hydrolysate and the minimal reactive dose of pyrogen of 0.01  $\mu\text{g.}$  The results indicate only roughly the amount of

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pyrogen involved since the standard of comparison is not completely purified and differences in the reactivity of pyrogen from different bacterial sources have been reported.<sup>18</sup>

Three to five rabbits were used for each test. The amounts of the substances indicated are the dosages per kilogram of weight. The volume of the purified pyrogen solution injected was 1 c.c. per kilogram; of the hydrolysate, 10 c.c. per kilogram.

The purified pyrogen was prepared by a modification of the method of Welch and co-workers<sup>15</sup> from *Pseudomonas aeruginosa*.

The pyrogen content of an enzymatic protein hydrolysate is shown in Table I. It contains over 0.01  $\mu$ g. of pyrogen per milligram of hydrolysate. Other enzyme preparations have contained as much as 0.01  $\mu$ g. in 0.1 mg. In repeated determinations with the same type of preparation, tests with only two or three dilutions were sufficient to establish the amount of pyrogen present. A large number of tests has been performed with the acid hydrolysates. The limitations of these data are shown by a typical experiment in which 5 mg. of hydrolysate gave a minimal reaction whereas 3 mg. did not.

TABLE I  
TEMPERATURE RISE PRODUCED IN RABBITS BY A PURIFIED PYROGEN AND BY A PROTEIN HYDROLYSATE (ENZYME)

PURIFIED PYROGEN ( $\mu$ G)	TEMPERATURE RISE (° C.)	PROTEIN HYDROLYSATE (ENZYME) (MG.)	TEMPERATURE RISE (° C.)
1000	2.0, 1.8, 1.1*	500	1.6, 1.2, 0.8
100	2.3, 2.0, 2.2	50	1.8, 1.7, 1.6
10	1.5, 2.3, 1.8, 1.6, 1.2	25	1.3, 2.0, 1.1
1	1.0, 2.4, 1.7	10	0.9, 0.6, 0.3
0.1	0.6, 1.5, 0.9	5	0, 0.7, 0.9
0.01	0.80, -0.1, -0.1†	1	0.3, 1.2, 0.9†
0.001	0.5, 0.4, 0.3†	0.1	0.3, 0.4, 0†

\*Two rabbits of this group died.

†A temperature rise exceeding 0.6° C. is indicative of pyrogens.<sup>15-17</sup>

*Preparation of Protein Hydrolysates.*—To obtain a pyrogen-free enzymatic digest, it was concluded on the basis of comparison of enzymatic hydrolysates of casein and human globin that the protein used should be essentially free of pyrogens. A pyrogen-free preparation was obtained from human red cells, collected aseptically and digested with fresh beef pancreas, precautions being taken to maintain sterile conditions throughout. Studies with this material were discontinued, however, because human globin is a deficient protein<sup>19</sup> and its availability is uncertain. Attempts to use this method with casein were unsuccessful because of its initial high content of pyrogen which could not be removed in subsequent operations. A small amount of pyrogen can be removed by Seitz filtration from water and from amino acid solutions, but it is difficult to accomplish when peptides are present as in enzymatic hydrolysates.

Since pyrogen-free proteins are not readily available, recourse was had to hydrolysis with acid, which destroys the pyrogen. Acid hydrolysates can readily be prepared by methods in general use<sup>20</sup> or those devised for preparing hydrolysates for bacterial nutrition.<sup>21</sup> Alkaline hydrolysates<sup>9</sup> were not considered desirable because of the racemization of the amino acids and the destruc-

tion of some of them. Since acid destroys all of the tryptophane, this essential amino acid must be added to acid hydrolysates. After the addition of tryptophane, preparations were tested for their ability to support the growth of rats,<sup>22</sup> since recent studies have indicated that the human requirements are similar to those of the rat.<sup>23, 24</sup>

After the acid used for hydrolysis was removed, bacterial contamination was avoided, as far as possible, to prevent the re-entry of pyrogen. The final hydrolysates were tested for pyrogens, the small amount of pyrogen usually present (about 0.01  $\mu\text{g.}$  in from 2 to 5 mg.) being removed by Seitz filtration. After filtration the pyrogen content was less than 0.01  $\mu\text{g.}$  per 100 mg. of hydrolysate; that is, 10 c.c. per kilogram of a 10 per cent solution gave no reaction in a rabbit.

A typical, dried casein hydrolysate contained 12.5 per cent total nitrogen, 66.2 per cent of which was amino nitrogen, 4.2 per cent moisture, and 2.3 per cent ash.

*Removal of Pyrogen:* The bacterial pyrogens are extremely soluble substances which appear to be polysaccharides.<sup>12, 13, 17</sup> In one case they have been reported to be nitrogen-free.<sup>13</sup> The purified pyrogen which we have isolated also has been largely polysaccharide in nature. Further study will probably provide a specific means for the destruction or removal of pyrogens from solutions, although none is available at present. None of the various methods tried for the removal of pyrogen has been adequate when a large amount of pyrogen was present. Filtration through asbestos pads (Seitz or Ertel) by the method of Co Tui and Wright<sup>12</sup> has been successful when the amount was small.

TABLE II

REMOVAL OF PYROGEN FROM VARIOUS SOLUTIONS AND PROTEIN HYDROLYSATES BY FILTRATION THROUGH A SEITZ PAD

SUBSTANCE	PYROGEN CONTENT ( $\mu\text{g.}$ PER 1,000 C.C.)	AREA OF SEITZ PAD* (SQ. CM.)
Purified pyrogen† in water	5,000	648
Casein hydrolysate (acid), 10%	300	65
Casein hydrolysate (enzyme), 10%	10,000	26,000
Purified pyrogen in 10% pyrogen-free casein hydrolysate (acid)	1,000	> 648

\*In actual practice the volume of solution which could be freed of pyrogen by passage through two pads 20 cm. in diameter (648 sq. cm.) was determined.

†A temperature rise exceeding 0.6° C is indicative of pyrogens.<sup>15-17</sup>

A summary of the results of the removal of pyrogen by filtration is given in Table II. Purified pyrogen, 5,000  $\mu\text{g.}$  dissolved in 1,000 c.c. of water, was removed by filtration in an Ertel filter<sup>13</sup> through two S-3 Seitz pads (648 sq. cm.). Additional experiments showed that this was the absorptive capacity of the pads. Each square centimeter of pad removed about 10  $\mu\text{g.}$  The Ertel No. 0 pads were no more effective than the Seitz pads. Co Tui and Wright<sup>12</sup> reported the removal of 100  $\mu\text{g.}$  of a less active pyrogen per square centimeter of the Ertel pad. Unfortunately these data were not directly applicable to protein hydrolysates. The pad area required to remove equivalent pyrogen from enzyme hydrolysates was at least twenty times as great as that required for pure pyrogen in water. The area required for acid hydrolysates was almost the same as for pyrogen

removed from water. In some experiments larger areas were required than for the example shown in Table II. That this was due to the amino acids and not to a difference in the properties of the pyrogens was shown by the experiment in which the purified pyrogen was added to an acid hydrolysate previously freed of pyrogen. Filtration was much less effective than with the equivalent amount of pyrogen added to water. The difference between acid and enzyme hydrolysates was probably due to the presence of polypeptides, since the former is 100 per cent hydrolyzed, whereas the latter is only 63 per cent hydrolyzed.

#### DISCUSSION

The method described for the quantitative determination of pyrogen is time consuming. Further study of the bacterial pyrogens may reveal specific properties on which a more rapid determination can be based. Such knowledge also may reveal more satisfactory means for their destruction or removal. From a practical standpoint, the quantitative removal of small amounts of pyrogens from complete hydrolysates is feasible, but their removal from partial enzyme hydrolysates is impractical. In the use of large filtering areas the possibility of loss of essential amino acids must be kept in mind.

#### SUMMARY

A technique based on the minimal reactive dilution is described for determining the bacterial pyrogen content of protein hydrolysates. The test is used in the preparation of protein hydrolysates (acid) which are freed of pyrogen by means of Seitz filtration. Results showed that partial hydrolysates (enzyme) could not be freed of pyrogen by Seitz filtration.

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# THE TITRIMETRIC DETERMINATION OF POTASSIUM IN TUNGSTIC ACID FILTRATES

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## INTRODUCTION

THE basis of several methods<sup>1-4</sup> for the estimation of potassium in serum and plasma has been its precipitation by the addition of the cobaltinitrite ion to the preparation. However, in such procedures a mixture of insoluble salt complexes, namely,  $K_2NaCo(NO_2)_6$  and  $K_3Co(NO_2)_6$ , is almost always formed which necessitates the determination of a conversion factor to obtain the true values for the amount of potassium in the precipitate. In the method to be reported in this communication it has been found that the potassium is quantitatively combined with the cobaltinitrite ion, and evidence is presented which indicates that the ratio between the potassium and the nitrite portions of the molecule is a constant quantity if the directions given for the determination are carefully followed. Consequently the titrimetric estimation of the nitrous acid by potassium permanganate gives a simple and accurate method for the measurement of potassium in the cobaltinitrite complex.<sup>1, 2</sup>

Although the complex of potassium and cobaltinitrite is soluble in tungstic acid filtrates, precipitation is essentially quantitative in the presence of 30 per cent ethyl alcohol. The method to be described in this report makes use of this fact, and therefore it is possible to determine potassium directly in tungstic acid filtrates of serum and plasma, as well as to determine other constituents such as chlorides or nonprotein nitrogen in aliquots of this same filtrate. In previous methods using tungstic acid filtrates, the cobaltinitrite was estimated colorimetrically.<sup>3, 4</sup> Since the potassium is determined titrimetrically in this procedure, the apparatus employed is simple and can be found in almost any clinical laboratory. With as little as 1 c.c. of serum or plasma, estimations of potassium may be made in triplicate. The method is relatively rapid and lends itself readily to routine clinical analyses; it has been used for this purpose with satisfactory results in our laboratory for the last year.

## REAGENTS

1. Stable tungstic acid solution.<sup>5</sup>
2. Sodium cobaltinitrite reagent.<sup>1</sup>
3. Ethyl alcohol, 95 per cent.
4. 0.01 N potassium permanganate, prepared by dilution just before use from a stock 0.10 N solution which is made by dissolving 3.158 Gm. pure potassium permanganate in 100 c.c. water.

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5. 0.01 N sodium oxalate prepared by dilution just before use from a stock 0.1 N solution which is made by dissolving 6.700 Gm. sodium oxalate (Sorenson's analytical reagent, dried over calcium chloride) in 1 liter of water.

6. 4 N sulfuric acid.

#### METHOD

*Precipitation of Protein.*—The protein in 1 c.c. plasma (or serum) is precipitated by adding 9 c.c. of stable tungstic acid reagent and allowed to coagulate for approximately thirty minutes, then centrifuged for twenty minutes at 1,500 r.p.m.

*Precipitation of Potassium.*—A 2 c.c. aliquot of the clear supernatant fluid (equivalent to 0.2 c.c. of plasma) is added to a clean\* centrifuge tube containing 1 c.c. 95 per cent ethyl alcohol. After mixing well, the solution is allowed to stand for five minutes to allow any microbubbles to disappear before adding 1 c.c. of the filtered sodium cobaltinitrite reagent slowly, drop by drop, agitating the mixture after each addition. After standing from forty-five to sixty minutes, the contents are centrifuged for twenty minutes at approximately 1,500 r.p.m. The clear supernatant fluid is sucked off slowly, using a fine bore tube drawn out to a J-shaped capillary, leaving about 0.2 c.c. of the fluid above the well-packed precipitate. The first portion of the 5 c.c. wash water is allowed to slide slowly down the side of the tube to prevent any disturbance of the precipitate and to rinse down any particles of the precipitate which might be adhering to the walls. Finally, the last portion of the water is used to wash the capillary pipette. To ensure mixing, the tube is then rotated carefully between the palms of the hands until the contents are a uniform color. The washing process is repeated four times with no more than from five to ten minutes of centrifuging between washes.

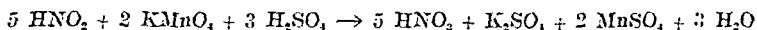
*Titration.*—The potassium permanganate solution is standardized just before use by adding 2 c.c. permanganate to 1 c.c. 4 N sulfuric acid and heating for from thirty to forty-five seconds in boiling water. A 2.5 c.c. sample of the standard 0.010 N sodium oxalate is added and the tube is heated in the water bath again and the excess oxalate titrated to a permanent pink color with the permanganate to be standardized. A blank, 0.025 c.c., the amount of permanganate necessary to give 1 c.c. of 4 N sulfuric acid added to 4 ml. water a permanent pink color, is subtracted from the total permanganate used. The normality of the permanganate is calculated by dividing the amount of permanganate required to neutralize the oxalate by the amount of oxalate employed (2.5 c.c.) and multiplying the result by 0.01.

For normal plasma (or serum), 2 c.c. of potassium permanganate are added to the precipitate; after intimately mixing with a fine-tipped glass rod, 1 c.c. 4 N sulfuric acid is added, rinsing down the glass rod at the same time. The tube is then placed in gently boiling water for from thirty to forty-five seconds. At the end of this time all of the precipitate should be dissolved and the solution a clear pink color. Either 1 or 2 c.c. of 0.010 N sodium oxalate, depending upon the amount of potassium in the sample, is added while the tube is still very hot. The contents of the tube are well mixed and then heated up in the

\*The centrifuge tubes may be cleaned with a warm 2 per cent solution of "Orvus" followed by thorough rinsing with distilled water. This removes all traces of grease which would tend to trap small amounts of the fine precipitate and thus increase the possibility of losing some of it during the washing procedure.

boiling water for at least one minute before titration with the 0.01 N potassium permanganate to a permanent pink color, using a 1 c.c. microburette or a 1 c.c. pipette with a very fine tip and graduated in hundredths.

*Calculation of the Factor for Converting the Titration Value to Milligrams of Potassium per 100 c.c.*—The addition of acid to the precipitate liberates the nitrite portion of the complex as nitrous acid. The reaction with permanganate then proceeds as follows:



If the permanganate is exactly 0.01 Normal, the amount of potassium is then given by the equation:  $([ (a - b) - d ] \times f)$  mg., where

$a$  = the actual volume of permanganate required in the analysis

$b$  = the blank (0.025 c.c.)

$d$  = the amount of standard 0.010 N sodium oxalate added.

$f$  = the factor for converting the titration value to milligrams of potassium per 100 c. c.

In Table I is shown how the value for  $f$  was obtained from the determination of potassium in solutions containing amounts ranging from 62.8 mg. to 10 mg. potassium per 100 c.c. when the normality of the permanganate was exactly 0.01. However, if the permanganate is not exactly 0.01 N, but has a normality of  $N^1$ , the fraction  $\frac{N^1}{0.01}$  should be applied to the volume  $(a - b)$  of permanganate used. The milligrams of potassium per 100 c.c. of the sample analyzed by this procedure is therefore:  $([ (a - b) \frac{N^1}{0.01} ] - d) f$

TABLE I  
CALCULATION OF THE CONVERSION FACTOR  $f$

(1) STANDARD SOLUTION OF K ADDED AS KCl MG. PER 100 C.C.	(2) $\left\{ (a - b) \frac{N^1}{0.01} \right\} - d$	(1) (2) $f$
62.8	1.951	32.2
47.1	1.447	32.5
47.1	1.390	33.9
40.0	1.219	32.8
40.0	1.200	33.3
35.3	1.082	32.6
31.4	0.997	31.5
23.6	0.736	32.1
23.6	0.745	31.7
23.6	0.715	33.0
20.0	0.621	32.0
20.0	0.619	32.3
20.0	0.662	30.2
20.0	0.614	32.6
20.0	0.604	33.1
15.7	0.480	32.7
15.0	0.460	32.6
15.0	0.478	31.4
11.8	0.356	33.1
11.8	0.358	33.0
10.0	0.306	32.7
Average		32.5 $\pm$ 0.8

## RESULTS

Using the value of  $f = 32.5$ , single analyses were done on a sample of dog plasma to which were added known amounts of potassium chloride with the results shown in Table II. It is possible, therefore, to recover potassium quantitatively when it has been added to dog plasma. The average difference between duplicates of forty-two determinations in which this procedure was used amounted to 0.5 mg. per 100 c.c. plasma. Therefore the level of potassium in the sample analyzed should be within 3 per cent of the true value.

TABLE II  
RECOVERY OF POTASSIUM ADDED TO DOG PLASMA

K FOUND (MG. PER 100 C.C.)	K ADDED (MG. PER 100 C.C.)	RECOVERY (MG. PER 100 C.C.)	DIFFERENCE (MG. PER 100 C.C.)
19.1	0.0	0.0	
23.1	4.0	4.0	0.0
23.2	4.0	4.1	+0.1
43.6	25.1	24.5	-0.6
44.2	25.1	25.1	0.0

In a series of thirty normal dogs the value for plasma potassium was found to vary between 13.0 and 19.8 mg. per 100 c.c. with an average value of 15.9 mg. per 100 c.c. Manery and Solandt,<sup>6</sup> using a slight modification of the Shohl and Bennett potassium method as described by Fenn and co-workers,<sup>7</sup> found that the concentration of potassium in plasma of forty-four normal dogs ranged from 12.2 to 20.0 mg. per 100 c.c. with an average value of 15.9 mg. (probable error,  $\pm 1.7$  mg.).

## SUMMARY

1. A titrimetric method for the estimation of potassium in tungstic acid filtrates of serum or plasma has been described. The procedure is also applicable to lymph and ascitic and cerebrospinal fluid.

2. Determinations may be done in triplicate on 1 c.c. of serum or plasma.

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## A COLORIMETRIC METHOD FOR DETERMINING AVAILABLE OXYGEN IN BLOOD

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**I**N PLANNING a clinical study of pulmonary anoxemia, one of the requirements was the collection of data showing the effect of different test conditions on the oxygen content of arterial blood. This requirement seemed to call for repeated arterial punctures, a procedure which is objectionable, if not impracticable. There remained only two alternatives: to use the so-called venous-arterial blood samples obtained from a vein after immersing an arm in hot water to speed up the circulation<sup>1</sup> or to use capillary blood samples from a pricked finger.<sup>2</sup> We used capillary blood samples in our determination of carbon dioxide<sup>3</sup> and found this entirely satisfactory. An inquiry into the differences of the oxygen content between arterial and venous blood under given controlled conditions was also contemplated, so the collection and care of venous blood has also been carefully considered. If the blood taken from the cubital vein or from a vein near the knuckles of the hand after immersion in hot water or from a finger prick is to remain a true sample for an oxygen content study, it must be protected from the atmosphere. With a finger-tip prick this was readily done by immersing the bleeding finger under oil.<sup>3</sup> The venous blood may be drawn with a syringe containing a little oil or attach the needle to a short rubber tube, the other end of this tube in turn attached to a glass tube running to the bottom of oil in a test tube, as suggested by Van Slyke and Cullen.<sup>4</sup> It was not considered advisable to allow any oil in a needle to be introduced intravenously into a human subject, so in collecting venous blood we took 6 c.c. in the usual way, but the needle point was always very promptly brought under the surface of the oil in a sample vial and 4 c.c. of the blood slowly expelled. The 2 c.c. withheld in the syringe were discarded on the assumption that the first portion entering the syringe would gather and hold all the atmospheric oxygen in the needle bore and the space between plunger and walls of the syringe. This is undoubtedly true in part, but the rush of the current from the very small orifice of the needle overcomes the plasma viscosity and carries through to the end of the plunger, thoroughly mixing the sample. There is also the matter of contamination from the oxygen dissolved in the oil. This will be taken up in the discussion of Table II.

Ferrous iron is very readily converted into ferric iron by molecular oxygen in a faintly alkaline menstruum but not at all in one that is acid. Ferric iron is very readily measured by converting it into any one of a large number of colored compounds. From an extended laboratory survey not only of iron, but also of other easily oxidizable substances, ferrous sulfate in a borax

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solution followed by sulfosalicylic acid was chosen as the most likely way to determine colorimetrically the oxygen in the blood both conveniently and quickly and on small samples such as could be obtained from a finger prick. Sulfosalicylic acid precipitates the blood proteins, getting them out of the way, and also combines with the ferric ion, giving a soluble compound which imparts a characteristic, stable, and consistent color to the solution with a color density directly related to the amount of ferric iron involved.

We now determine the available oxygen in the blood by mixing 0.1 c.c. blood sample with 0.1 c.c. ferrous iron solution in 1.5 c.c. borax solution, then introducing this mixture into 2 c.c. 30 per cent sulfosalicylic acid, making it up to 10 c.c. with water, filtering through iron-free paper, and measuring in a calibrated scopometer<sup>5</sup> the density of the color which develops. The oxygen content is then found opposite the shutter scale reading on a calibration chart.

#### EXPERIMENTAL

*Reagents.*—The required alkalinity in this test is not confined to a narrow range of pH, and the anions controlling the pH need not be limited to those of borax. Many alkalies in various concentrations have been successfully used, but we have finally settled on the convenient routine of 1.5 c.c. of 20 Gm. borax ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) per liter of water. The borax solution is fully aerated at atmospheric pressure and room temperature. The  $\text{Fe}^{++}$  reagent is added to the borax solution immediately after the blood sample. This reagent, likewise, need not be narrowly confined to a given concentration or anion, and in our laboratory it is prepared as follows: 500 mg.  $\text{FeSO}_4 \cdot \text{H}_2\text{O}$  are dissolved in 10 c.c. water, a little celite (inert filter aid) is added, and the solution is brought to a boil and kept simmering a few moments; it is then poured into a conical centrifuge tube, covered with mineral oil, and centrifuged. This reagent under oil is ferric-free and will remain so for days. However, it is well to make it up fresh each day an oxygen determination is done since the possibilities of  $\text{Fe}^{++}$  oxidation to  $\text{Fe}^{+++}$  by air contamination are considerable.

*Anticoagulants.*—Most blood comes to the laboratory with oxalate added as an anticoagulant. These samples are proper for acid hematin and the manometric procedures but give faulty values by the new colorimetric method since the  $\text{C}_2\text{O}_4$  ion interferes with the oxidation of iron. Fluorides were also found unsatisfactory, sometimes depressing the oxygen values 20 per cent. The only anticoagulant found which was at all suitable proved to be heparin. The heparin used comes in 100 mg. vials.\* A 1 per cent stock solution is made up and kept in the cold chest; this is rediluted as needed to measure out 0.3 mg. per 5 c.c. blood sample conveniently.

*Apparatus.*—It is obvious that during the interval when the oxidation of the  $\text{Fe}^{++}$  takes place no atmospheric oxygen must reach the reaction mixture. This we failed to accomplish in the ordinary test tube, using a layer of mineral oil to exclude the air, but we now do it without oil very conveniently by exchanging the test tube for a closed reaction chamber with a funnel cup joined to it by means of a stopcock to admit the sample and reagents and a rubber tube with mercury and mercury-levelling bulb below by way of another stopcock

\*From the Connaught Laboratory of Toronto University.

for controlling the flow of fluids between the cup and the chamber. Fig. 1, a drawing of the apparatus, is almost entirely self-explanatory. The capacity of the reaction chamber is 3.5 c.c., and of the cup, 5 c.c., the latter calibrated to 4 c.c. in 0.5 c.c. intervals from the stopcock up. The cup is 65 mm. long and 16 mm. in diameter. The contraction of the funnel cup along toward the stopcock by which it is joined to the reaction chamber forms a 2 mm. capillary bore about 7 mm. long. The two stopcock barrels are 38 mm. long and have a 15 mm. inner diameter across the wider end. The stopper projects some 10 mm. at the tapered end and has a groove just adjacent to the cock over which a rubber washer is slipped to keep the stopper from coming loose unexpectedly. The bore

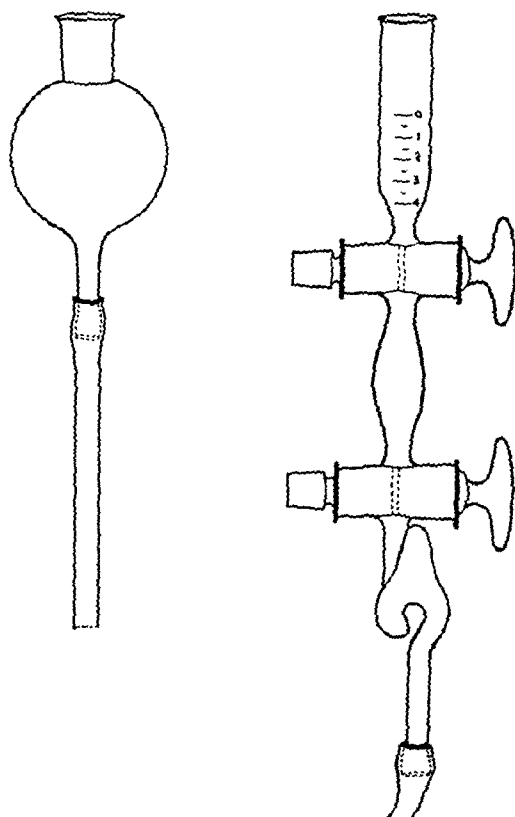


Fig. 1.

of the stopper is 2 mm. It is important that the upper stopper does not form a ledge upon which the blood may lodge; the bore should, therefore, be the same as the exit orifice of the cup capillary or a trifle more. The orifice of the upper stopper bore must not be bevelled. The glass tube by which the rubber tube is attached to the lower stopcock is looped and the top of the loop is expanded to form a trap (10 by 12 by 12 mm.) which catches any gas that may accidentally enter the mercury. Two seats are provided for the levelling bulb: in the upper seat the mercury is allowed to come to about the 1 c.c. mark in the cup; in the lower seat the mercury comes into the taper just above the lower stopcock.

It is very important that the apparatus be as clean as possible. It may be cleansed with water, alkali, or acid, or sometimes with chromic-sulfuric clean-

ing mixture, by drawing the cleaning fluid down through the cup into the reaction chamber, even to the trap if necessary, and then forcing it up again into the cup, all by changing the level of the mercury reservoir. The cleaning fluids must not add ferric iron as a contaminant. If the chamber is smeared with blood residues, it is most easily cleaned with a strong alkali, for instance, 2 c.c. 10 per cent NaOH, raising and lowering the levelling bulb so that the mercury will scrub the objectionable matter into the cup, from which it is flushed out with water by a pipette with a rubber bulb, or by the suction of a water pump. The apparatus must be free from protein, fat, and grease smears since these occlude oxygen and it takes only 0.001 c.c. oxygen to make an error of 1 volume per cent. The alkali should be rinsed out completely with iron-free water and finally with very dilute acid (0.05 N  $\text{H}_2\text{SO}_4$ ). When the apparatus is put away, it should be filled with this dilute acid with the lower stopcock closed, the upper open, and the levelling bulb in its lower seat.

#### PROCEDURE

To determine the oxygen capacity of the blood sample, assure the complete saturation of blood by rolling it over the glass surface of an open vial in such manner that it does not churn in air bubbles and does not concentrate the hemoglobin appreciably by moisture evaporation. Have the apparatus set to receive the subsample. Pour a little more than 4 c.c. borax solution into the funnel cup. *Insure the absence* of minute air bubbles by drawing some of the solution into the reaction chamber and by gently shaking, turning the stopper and by forcing the mercury up and down. Bring the mercury column just up to the top of the upper stopcock bore, adjust the volume of the borax solution to exactly 4 c.c., and then let about 0.1 or 0.2 c.c. come under the upper stopcock. Close the lower stopcock but leave the upper one open. Draw the blood up into a pipette having two graduation marks, the so-called Ostwald-Van Slyke pipette. When oxygen content is determined (in which case the sample is kept under oil), keep the tip of the pipette away from the oil-water interphase as well as from the bottom and wipe the oil from the pipette. Lower the pipette down into the borax solution so that the tip comes a little above the stopcock. Let the blood run slowly into the borax solution. The blood will trickle through the bores and settle on the surface of the mercury. If the blood comes up over the tip of the pipette, it may follow the pipette when the pipette is withdrawn. Do not let the blood back up unnecessarily; an occasional quick turn of the lower stopcock is helpful in avoiding this. Introducing the blood sample does require care, but is not at all troublesome; good technicians do it neatly and quantitatively on the first trial. Immediately after the blood has been measured into the borax solution, cover it with 0.1 c.c. ferrous reagent. For this purpose also use a pipette with two graduation marks. Draw the reagent into the pipette from a few millimeters below the oil-water interphase, wipe off the oil, and then let the reagent flow out at a moderate rate just over the blood. Blood sample and ferrous reagent must not come into contact with the atmospheric oxygen. As the  $\text{FeSO}_4$  enters the borax solution,  $\text{Fe}(\text{OH})_2$  precipitates as a very fine blue-green cast suspension. When the pipette is withdrawn, some of the flakes may be carried up by the current. Since there will be ample  $\text{Fe}(\text{OH})_2$  to meet

all the active oxygen present, these may be discarded. Those reaching the top will become contaminated and contribute to the ferric iron if they again settle; therefore, flush the mass of  $\text{Fe}(\text{OH})_2$  suspension on the bottom of the cup into the chamber before the floating  $\text{Fe}(\text{OH})_2$  particles settle. Draw the  $\text{Fe}(\text{OH})_2$  down into the reaction chamber with the borax solution so that 1.5 c.c. borax solution is measured in as accurately as can be done by the cup's graduation marks. Close both stopcocks, draw off the excess borate solution, and mix by gentle shaking. The reaction is complete within the first minute, but we let it stand for two minutes.

Any available oxygen in the reaction chamber will convert its equivalent mass of  $\text{Fe}(\text{OH})_2$  to  $\text{Fe}(\text{OH})_3$ . Rinse the cup with three portions of water and *pipette*\* 2 c.c. 30 per cent sulfosalicylic acid into the cup, raise the levelling bulb, and open first the upper stopcock and then the lower so as to admit the fluid into the acid *slowly*. As the reaction mixture flows into the cup, stir it constantly with a slender rod until it is all well up in the cup. Draw off the colored, turbid fluid with a pipette having a rubber bulb and transfer it to a 15 c.c. graduated conical centrifuge tube. Rinse the cup with small portions of water until the meniscus of the fluid accumulating in the conical tube is just at the 10 c.c. mark, add a pinch of celite (filter aid), shake well, let stand a few moments for the precipitate to organize, and then filter through iron-free paper. Read the *perfectly clear*† iron-sulfosalicylate color density in a calibrated scopometer, and on its calibration graph note the volume per cent oxygen corresponding to the shutter scale reading.

#### CALCULATION

One molecule oxygen oxidizes 4 atoms ferrous iron; 1 mg. iron, therefore, is equivalent to 0.144 mg.  $\text{O}_2$ , the mass of oxygen occupying 0.1 c.c. at  $0^\circ \text{C}$ . and 760 mm. Hg. The blood oxygen would seldom reach 30 vol. per cent, and that may be taken as a high level for standards; this would be represented by 0.3 mg. Fe per cubic centimeter. If 259.2 mg. ferric ammonium sulfate crystals ( $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ) are dissolved in water, made acid with 4 c.c. 30 per cent sulfosalicylic acid, and diluted to just 100 c.c., it will form a stock solution of such a concentration that when 1 c.c. of this is taken and 2 c.c. 30 per cent sulfosalicylic acid and 7 c.c. water are added, it will give a 10 c.c. colored solution with a tint and color density which are identical to a filtrate from a blood sample containing 30 vol. per cent available oxygen. This stock standard properly diluted to give lower oxygen levels was used to calibrate the instrument and may be used to make comparison solutions for visual colorimetry by the Duboseq type instrument. These standards are easily made up and keep very well.

Since the reagents are all aerated at atmospheric pressure and room temperature and the apparatus is rinsed with aerated water, there will be molecular oxygen to oxidize  $\text{Fe}^{++}$  besides that from the blood sample. A "blank" must, therefore, be done to account for this oxygen and such other consistent errors as may be involved. From the literature and from our solubility determinations

\*The salicylic acid is measured with a pipette since marked differences in acidity affect the color density of iron salicylate solutions. There must not be any air bubbles introduced with the acid.

†Refilter the colored solution if a turbidity is visible in it.

of oxygen dissolved in water, the blanks in the regular procedures as herein described should be close to 9.8 volume per cent, but they usually fall between 8.6 and 9.3. To establish the blank, and this should be done frequently, proceed exactly as in the blood oxygen determination described, only omitting the 0.1 c.c. blood. Find the equivalent blood oxygen volume per cent and subtract this blank from that found in the regular routine procedure. When the oxygen is determined on aerated blood, to give the oxygen capacity or the normal active hemoglobin, the sample will carry an extra 0.5 vol. per cent which is added to the blank before the subtraction.

#### RESULTS

It has been assumed that the Van Slyke-Neill manometric method<sup>6</sup> gives the true available oxygen content. The Van Slyke-Neill method gives as true values for oxygen as can be obtained in blood at the present time. As far as precision is concerned, one is not justified in seeking another procedure, but the manometric method calls for special skill, requires more blood than is sometimes convenient or available, and the apparatus is prone to unaccountable time-wasting leaks. The Van Slyke-Neill manometric blood oxygen method is a general approved means of standardizing hemoglobin determination methods; by the same token the oxygen capacity method for blood may be standardized by a hemoglobin determination if the hemoglobin is assured as being all normal and saturated with oxygen at proper temperature and pressure. For these reasons we have chosen to compare our new method with that published by Van Slyke and his co-workers and also with the hemoglobin in the samples as determined in our laboratory.

The acid hematin method<sup>7</sup> for determining hemoglobin has been very satisfactorily applied in our laboratory for more than twenty years. This is done by merely diluting 0.1 c.c. blood with 20 c.c. 0.1 *N* HCl and reading the color density twenty minutes after the dilution in a calibrated scopometer. Some modification of this way of determining hemoglobin is followed in most clinical laboratories. Time curves show that the maximum acid hematin concentration does not occur before the end of twenty minutes; the change between twenty and sixty minutes at room temperature is very little, if any.\* The instrument is calibrated and the calibration occasionally checked with blood of known iron content. Because of the simplicity and the small samples required, there were more comparisons of the new oxygen method with this hemoglobin method than with the Van Slyke-Neill procedure in the first months of the program. This is justified by the close agreement between the Van Slyke-Neill method and the acid hematin values. The average deviation from the mean of duplicates by the former is 0.177 and by acid hematin ( $Hb. \times 1.34$ ) it is 0.166 vol. per cent, and the agreement between the two methods is expressed by  $\pm 0.3$  vol. per cent, which is derived from four groups of analyses in which the numbers of samples were 127, 38, 123, and 157 and their average deviations from their means, 0.36, 0.23, 0.312 and 0.275.

Where there was a lack of agreement, the Van Slyke-Neill method more often checked the acid hematin than did our new procedure, but this has not

\*Increase in  $O_2$  from twenty to eighty minutes averages 0.16 vol. per cent; the average deviation from the mean at twenty minutes is 0.04, and at eighty minutes, 0.07.

been an invariable rule as the selected samples in Table I indicate. Since each method has a lesser deviation among duplicates than between any two of the three procedures, the differences by methods must be due to some inherent quality of the blood sample if bad checks in duplicates do not disqualify the data as due to manipulative error. The first four are typical of discordance; the next two are caused by a known inherent quality in the sample (namely, methemoglobin).

TABLE I

ACID HEMATIN (VOL. %)	COLORI- METRIC (VOL. %)	MANO- METRIC (VOL. %)	ACID HEMATIN (VOL. %)	COLORI- METRIC (VOL. %)	MANO- METRIC (VOL. %)	ACID HEMATIN (VOL. %)	COLORI- METRIC (VOL. %)	MANO- METRIC (VOL. %)
20.1	19.2	19.7	29.5	27.2	27.2	18.5	20.3	20.0
18.5	17.5	18.7	21.3	20.5	20.8	20.2	20.3	21.0
19.6	18.4	19.5	19.4	20.0	19.9	17.2	17.3	17.9
23.4	22.7	23.5	19.1	19.8	19.8	17.6	17.7	18.6

The acid hematin gives all the hemoglobin, and there may be some of this present which does not carry oxygen (methemoglobin). This discrepancy is a measure of the inactive hemoglobin. Blood samples should be examined promptly for available oxygen since the hemoglobin may change on standing. Methemoglobin in excess of 0.5 vol. per cent is not often encountered in random fresh blood samples,<sup>8</sup> but if these samples are set aside for some days, methemoglobin may form. The following samples were in good agreement between the acid hematin and direct oxygen determinations on the freshly drawn samples when they were put away in the cold chest, but on standing the oxygen values decreased to yield the following in these three sets: 15.2 to 14.0 vol. per cent; 15.6 to 14.8; 15.6 to 12.5. Sometimes the available oxygen remains constant or increases slightly as water evaporates, as in the following: Fresh blood sample, by the new method, May 14, 15.8 vol. per cent; May 17, 15.7; May 18, 15.9; May 19, 16.3; May 21, 16.5; May 24, 16.6. The acid hematin determination on the fresh blood sample gave a value of 15.6 vol. per cent. Three blood samples have also been encountered in which the oxygen content decreased on standing but was fully recovered again on aeration, a spontaneous deoxygenation. Methemoglobin does not vitiate the oxygen determination by the Van Slyke method nor by the new procedure, so that both methods may also be used in estimating the portion of hemoglobin which has been deprived of its oxygen transport capacity.<sup>9</sup> A methemoglobin blood preparation was made by treating a normal blood with ferrieyanide. One cubic centimeter of this preparation was then added to 2 c.c. of an aerated normal blood sample and the oxygen determined. The oxygen in this sample by the new method before adding the methemoglobin preparation came to 19.6 vol. per cent and to 19.2 by the Van Slyke-Neill method; that calculated from acid hematin hemoglobin was 19.6. Two-thirds of their average would be 13.0. By the actual determinations after mixing, they came to 13.6, 13.6, and 17.9, using the same methods and following the same order. In this set the acid hematin value minus the direct oxygen determinations comes to 4.3 vol. per cent; this value divided by 1.34 gives 3.2 Gm. methemoglobin per 100 c.c. of the mixture.

Oxygen was determined on many kinds of blood from the very beginning of this general problem, but until the method was believed to be in its final form

and reliable, most of the samples examined were fully aerated. These data on aerated blood are summarized in Table II. The time periods are arranged in chronological order. The first period includes many of the experiments done during the development of the method itself. Subsequent to this period there have been no essential changes in chemistry nor manipulation. The second period is noteworthy in that it was a flawless run of both colorimetric and manometric determinations on fresh bloods with remarkable agreement between the two procedures.\*

TABLE II

COMPARING THE NEW COLORIMETRIC METHOD FOR OXYGEN IN BLOOD WITH THE VAN SLYKE-NEILL AND/OR THE ACID HEMATIN METHODS ON AERATED BLOOD

PERIOD	NUMBER OF SAMPLES	MEAN OF DIFFERENCES BETWEEN TWO METHODS	AVERAGE DEVIATION FROM THE MEAN DIFFERENCE		PER CENT OF SAMPLES IN WHICH THE AGREEMENT CAME WITHIN	
			FOR ALL SAMPLES	FOR THOSE WITHIN $\pm 1.0$	$\pm 0.6$	$\pm 1.0$
I	416	+0.7	0.61	0.48	69	87
II	25	+0.036	0.10	0.10	100	100
III	220	+0.01	0.37	0.33	87	96

The subsequent routine determinations on aerated blood indicate that the new procedure gives results which agree with manometric determinations to within 0.5 vol. per cent in more than 85 per cent of the cases.

The application of the method to venous blood is summarized in more detail in Table III. These samples were obtained from volunteers among friends of the department in the Home Office, and we are duly grateful to them for the interest shown in our work. In most instances they came directly from their desks; a few were having metabolism tests and the samples were taken under basal control. They were all presumably normal subjects. The two tests were done simultaneously, the Van Slyke-Neill manometric method by Miss Weakley and the new colorimetric by Mr. Huizer. In this series we were also concerned with the oxygen exchange between the hemoglobin and the oil used to protect the blood from the oxygen in the atmosphere. The sample was protected from the atmosphere but not from the oxygen dissolved in the oil. Kubie<sup>10</sup> accepts the protective function of oil and explains it as due to the very slow diffusion of gas through the oil, not to exclusion from lack of solubility. He bubbled oxygen from a cylinder into mineral oil for a long period and in that way determined the capacity of the oil as 0.134 c.c. oxygen per cubic centimeter. We have determined the oxygen content of our oil supply on two days, four months apart, by both methods. The average for the manometric method came to 2.40 and 2.42 vol. per cent and by the colorimetric, 2.3 and 2.8, making an average of 2.47 vol. per cent for all the determinations. Fully aerated blood passing through this approximately fifth oxygen-saturated oil did not lose any oxygen, but samples containing any deoxygenated hemoglobin picked up oxygen. The oxygen in the samples reported in Table III were determined at once by the two

\*The determinations in this series by the new method were done by Dr. F. Schattner and Mr. J. Huizer, and those by the Van Slyke-Neill methods, by Miss Mary C. McCarthy and Miss Mary L. Weakley. Grateful acknowledgment must also be made for the splendid and kindly cooperation of Dr. William Antopol of the Laboratory of the Newark Beth Israel Hospital, for supplying many blood samples.



TABLE III

	IMMEDIATE			THROUGH OIL BY DROPS			GAIN FROM OIL		OXYGEN SATURATION			AERATED BLOOD			L/M P (%)	L/N Q (%)	L/O R (%)	N/M S (%)	
	A (VOL. %)	B (%)	C (%)	D (VOL. %)	E (%)	F (%)	G (VOL. %)	H (VOL. %)	I (VOL. %)	J (%)	K (%)	L (VOL. %)	M (VOL. %)	N (%)					O
H.S.	6.9	6.74	102.4	8.9	8.77	101.0	1.0	2.0	15.8	40	39	17.3	17.92	17.2	-	96.6	100.5	-	96.2
H.S.	6.5	6.19	105.0	7.9	7.40	106.5	1.4	1.2	16.5	39	38	17.4	18.10	17.6	18.0	96.2	99.0	96.6	97.2
H.S.	8.8	9.17	96.2	10.0	9.97	100.3	1.2	0.8	15.9	55	58	16.6	16.60	17.0	16.8	100.0	97.8	99.0	102.3
J.C.	6.0	5.73	104.6	7.5	7.57	99.3	1.5	1.9	18.2	33	31	20.8	22.22	20.5	-	94.1	101.5	-	92.3
M.G.	5.2	4.60	113.0	6.8	6.40	106.2	1.6	1.8	16.2	32	28	17.7	18.11	17.6	-	98.0	100.5	-	97.2
T.L.	6.5	6.47	100.5	8.1	7.96	101.5	1.6	1.5	17.1	38	38	17.7	18.69	17.6	-	94.8	100.5	-	94.2
M.A.	4.9	4.49	109.0	6.1	5.59	109.0	1.2	1.1	15.3	32	29	15.8	16.23	16.3	-	97.5	97.0	-	100.4
M.A.	6.1	5.64	108.0	-	-	-	-	-	15.3	40	37	15.6	15.30	15.3	15.3	102.0	99.0	102.0	100.0
H.M.	8.7	8.98	97.0	10.1	10.28	98.2	1.4	1.3	20.9	41	43	21.9	21.77	21.5	-	100.5	101.8	-	98.8
F.K.	9.5	9.97	97.4	10.7	10.78	99.2	1.2	1.0	20.8	46	47	22.3	23.03	20.0	22.0	97.2	107.2	101.3	90.5
M.K.	6.3	9.54	113.6	7.5	6.90	108.6	1.2	1.4	18.0	35	30	19.8	19.80	18.0	19.1	100.0	110.0	103.5	90.6
J.B.	12.3	12.99	94.8	13.7	13.74	100.0	1.4	0.7	19.4	63	67	20.9	20.80	21.2	20.6	100.5	98.6	101.3	101.9
P.P.	9.3	8.90	104.3	9.8	10.16	96.2	0.5	1.2	18.8	50	47	19.5	19.65	18.3	19.6	99.2	103.7	99.5	95.8
P.P.	7.9	7.30	108.0	9.1	8.55	105.5	1.2	1.4	20.0	39	37	21.5	20.96	20.0	21.0	102.5	107.4	102.4	95.8
J.C.	12.0	12.27	97.6	13.4	13.57	98.6	1.4	1.2	18.7	62	66	19.6	20.70	19.8	20.4	95.0	99.0	96.2	95.7
E.B.	9.0	9.10	99.0	9.8	9.97	98.6	0.9	0.8	19.6	46	51	20.5	21.20	20.7	21.5	96.8	99.0	95.2	97.7
R.W.	13.4	13.90	96.4	15.4	15.0	102.5	2.1	1.1	19.3	69	72	20.4	21.10	20.5	20.8	96.8	99.8	98.0	97.2
M.R.	7.1	7.00	101.5	7.8	7.94	98.4	0.7	0.9	19.5	36	39	20.5	20.97	20.4	20.6	98.0	100.2	99.8	97.4
A.R.	6.5	6.49	100.2	7.6	7.09	99.0	1.1	1.2	17.1	38	38	18.1	18.37	17.8	17.4	98.6	101.7	104.0	97.2
A.R.	3.9	3.98	98.0	5.8	5.09	113.5	1.9	1.1	16.5	24	24	16.7	16.85	17.5	16.6	99.0	95.5	100.5	103.6
W.F.	2.9	2.68	108.0	4.3	3.84	111.8	1.4	1.2	19.5	15	14	20.9	20.50	20.9	20.8	102.0	100.0	100.5	102.0
D.C.	10.8	11.60	93.0	12.5	12.5	100.0	1.7	0.9	20.5	52	55	22.7	22.50	21.9	22.4	101.0	103.5	101.2	97.3
S.C.	13.8	14.05	98.8	14.3	14.4	99.5	0.4	0.4	18.8	74	74	20.4	21.05	20.5	20.9	97.2	99.8	97.7	97.4
N.S.	2.7	2.69	100.5	3.7	3.36	110.0	1.0	0.7	16.5	16	16	16.8	17.57	17.2	17.6	95.5	97.8	95.5	97.8
S.S.	12.8	13.30	96.5	14.0	14.5	96.5	1.2	1.2	18.0	71	74	19.1	19.70	19.3	20.6	97.0	99.0	92.8	98.2
W.S.	8.8	8.78	100.0	9.7	9.67	100.0	0.9	0.9	19.4	45	45	20.0	20.07	20.4	20.0	100.0	98.0	100.0	102.0
A.W.	9.8	10.29	95.0	10.7	9.67	110.8	0.9	-0.5	18.5	53	56	18.5	18.50	19.2	19.0	100.0	96.4	97.5	103.7
J.H.	10.4	10.67	97.5	11.8	11.58	102.0	1.4	0.9	18.8	57	57	19.4	19.70	19.4	19.6	98.6	100.0	99.0	98.5
J.H.	7.3	7.50	97.5	8.7	9.06	96.0	1.4	1.6	19.3	38	39	-	-	-	-	-	-	-	-
J.H.	11.0	11.06	99.6	12.2	12.30	99.2	1.2	1.1	17.3	64	64	17.3	17.40	17.8	17.2	99.6	97.2	100.5	102.0
J.H.	12.2	11.88	102.5	13.2	13.36	98.8	6.8	1.5	17.6	70	68	18.5	19.20	19.0	19.2	96.5	97.2	96.5	99.0
Average			100.4			102.3													
			100.2			101.9													
Difference																			
Mean	0.040			0.050								0.150							
Average deviation	0.307			0.236								0.335							

methods. The "immediate" oxygen values are given under *A* and *B*. When the "immediate" oxygen had been determined, a syringe tip was put into the vial, some oil was drawn in and then the blood. The blood was then slowly pressed out in as small drops as possible under oil into another vial. This was repeated. Every caution was taken to keep the sample from direct contact with the atmosphere. Two subsamples were then taken and the "by drops through oil" oxygen determined and recorded under *D* and *E*. The increment between these two sets is an index of the contamination from the oil. The initial oxygen contribution to the blood sample from the oil, even also that from the empty needle as the blood is drawn from the vein, is probably no more than this increment; nevertheless it is real, averaging 1.21 mg. vol. per cent for the colorimetric column (*G*) and 1.18 for the manometric column (*H*) in Table III, with an average deviation of 0.28 from the mean for both columns, a possible contamination of the order of 10 per cent.

The main purpose of the venous blood analysis gathered in Table III was again to compare the new method with that of Van Slyke and Neill on the kinds of blood samples encountered in the clinical laboratory and used in physiologic investigations. The colorimetric data are listed under *A*, *D* and *L*; the manometric, under *B*, *E* and *M*. The hemoglobin was determined by acid hematin and read directly as equivalent oxygen volume per cent on a calibration chart; the hemoglobin in grams was multiplied by 1.34. The data for the blood as drawn are listed under *I* and those after aeration, under *N*. The iron<sup>11</sup> was also determined on the aerated blood and the milligrams per 100 c.c. multiplied by 400 to give equivalent volume per cent oxygen; these are listed in column *O*.

From *I* and *A* and *B* the oxygen saturation of the venous hemoglobin is calculated and listed under *J* for the new method and under *K* for the Van Slyke-Neill figures. These saturations cover a rather wide range, from 14 per cent for W. F. to 74 per cent for S. C., much wider and lower than those reported by Lundsgaard and Möller,<sup>12</sup> whose lowest saturation was 48 per cent and highest 96 per cent with a median at 77 per cent instead of 41 as in Table III; 78 per cent of their cases fall between 60 per cent and 90 per cent. In our list one-half of the cases fall between 38 and 58 per cent saturation. Any further comments on this matter will be left for the time being and taken up again when the data now accumulating in this area are published.

The determined volume per cent by the new colorimetric method (*A*, *D* and *L*) were multiplied by 100 and then divided by the values obtained with the Van Slyke apparatus (*B*, *E* and *M*) to give the percental ratios in columns *C*, *F*, and *P*. The small divergence of these *per cents* from 100, averaging *C*, 0.4; *F*, 2.3; and *P*, 1.6; prove the two methods to be in tolerable agreement. If one goes over Table III critically, one may cull values that may be discredited (those in italics) as, for instance, the case of N. S. with *M*, *N*, *O* values within a spread of 0.4 vol. per cent and the *L* value 0.6 below their average. In this instance the value by the new method is conceded as most likely too low; in four other subjects, on the other hand, the manometric figures are thought rather high if credence be not denied the iron and acid hematin readings (J. C., T. L., F. K.). Neither the acid hematin nor iron method is infallible. In the samples

from F. K. and M. K. the acid hematin figures ( $N$ ) are the same as those before aeration ( $I$ ), which is not in accord with experience. If due allowances are made for this way and the averages are recalculated with the questioned cases omitted, these figures come closer to 100 and are entered at the bottom of Table III. The improvement by thus selecting the cases is not significant. When the new method is compared with the acid hematin and the iron procedures in the same manner, we get columns  $Q$  and  $R$  by dividing  $I \times 100$  by  $N$  and  $O$ . These averages come to 100.2 and 99.2 and are again in excellent agreement. Aeration of the samples seemed to enhance the oxygen values by the manometric method, for which reason the percentile ratio of acid hematin/manometric methods were calculated and listed in column  $S$ . The average of  $S$  agrees well with that of  $P$ . A specific effect of aeration on one of the oxygen determinations may be real but is quantitatively insignificant for the purpose and therefore dismissed for the present. The two methods were also compared by their differences in volume per cent,  $A-B$ ,  $D-E$ , and  $L-M$ . The average deviations from the mean differences are given at the bottom of Table III. The eight italicized oxygen values are omitted in these averages. The range of those used are from  $-0.80$  to  $+0.76$ . Two samples among those omitted would extend the range from  $-1.40$  to  $+1.00$ . Less than 15 per cent of the cases exceed a difference of 0.5 vol. per cent, and the average difference of all eighty-three pairs in this table is less than 0.3 vol. per cent.

#### SUMMARY

A colorimetric method for the determination of available oxygen in 0.1 c.c. sample of blood from a vein or finger prick is presented. The method depends on the oxidation of ferrous iron by the oxygen of the blood sample to ferric iron and then combining this ferric ion with sulfosalicylic acid to yield a characteristic and consistent color whose density under the conditions of the test bear a direct relation to the amount of oxygen in the sample. The oxidation of iron takes place in a solution made alkaline with borax in an apparatus designed to protect it from atmospheric oxygen. The sulfosalicylic acid prevents further oxidation and removes the proteins. The color density may be compared with known ferric sulfosalicylate solutions in a visual instrument of the Duboseq type or may be measured in a calibrated photoelectric instrument like the scopometer. Oxygen values by this method have been compared with simultaneous determinations with the Van Slyke-Neill manometric method and with oxygen equivalents calculated from hemoglobin and iron determinations. The agreements of the several methods are of the order of  $\pm 0.5$  vol. per cent in 85 per cent of the cases.

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## BOOK REVIEWS

**Atlas of the Blood in Children.** By *Kenneth D. Blackfan, M.D.,* Late Thomas Morgan Rotch Professor of Pediatrics, Harvard Medical School; Late Physician-in-Chief, Infants' and Children's Hospitals, Boston; and *Louis K. Diamond, M.D.,* Assistant Professor of Pediatrics, Harvard Medical School; Visiting Physician and Hematologist, Infants' and Children's Hospitals, Boston; with illustrations by *C. Merrill Leister, M.D.,* Associate Pediatrician, St. Luke's Hospital, Bethlehem, Pa., and Allentown General Hospital, Allentown, Pa. The Commonwealth Fund, New York, N. Y. Price \$12.00. Cloth with 334 pages and 70 plates.

The seventy color plates about which this atlas is built are unquestionably the finest reproductions of peripheral blood films stained with Wright's stain that have so far been printed as a collection in this country. They are at least the equal of the excellent color plates which appeared in Downey's *Handbook of Hematology*. The illustrations were painted by Dr. C. Merrill Leister; 560 separate plates, each reproduced in eight colors, were made for the seventy full-page plates. Reproductions of color tone, of nuclear structure, and of cytoplasmic inclusion bodies are amazingly accurate. Eosinophilic and neutrophilic granules in several instances appear slightly diagrammatic, but this is a minor objection. A student or a physician trying to identify blood cells on a slide in which young or abnormal forms of cells are present would receive greater help from this atlas in his attempt to identify the cells which puzzle him than from any other source now available.

In addition to the color illustrations, there are a number of charts, 144 pages of text, and a comparatively short bibliography. The text itself, written by Drs. Blackfan and Diamond, is characterized by a simplicity and clarity of expression which is unusual in hematologic writings. Following a brief description of the blood in infancy and childhood, the authors discuss the etiology, the clinical manifestations, and diagnosis and treatment of the anemias, the diseases involving white blood cells, and the purpuras. For each dyscrasia, one or two cases histories, carefully selected to illustrate problems of diagnosis and treatment, are given. While the book concerns itself primarily with hematologic abnormalities in children, much of the information will prove valuable to the internist as well as to the pediatrician and student. It should be pointed out, however, that it does not purport to be a complete text of blood disorders. The lymphomas other than lymphatic leucemia and the hemorrhagic diseases other than purpura are discussed very briefly since they do not cause characteristic cytologic changes in the peripheral blood. Discussion is limited entirely to conditions with which the authors have had close personal experience.

While the reviewer does not wish to detract in any way from the impression he hopes to give of the book's fine qualities, he should record a few omissions

which seem unfortunate. Nothing is said in the section devoted to the treatment of congenital hemolytic anemia of the possible danger of accelerating hemolysis by giving transfusions prior to splenectomy. Sicklemia, or the sickle-cell trait, is not sharply differentiated from sickle-cell anemia. Most readers would appreciate knowing the percentage incidence of the various types of leucemia seen by the authors in their wide experience. The appearance of the megakaryocytes in the bone marrow of children with primary thrombocytopenic purpura is not described; many hematologists regard this cytologic change as moderately specific. Auer bodies are neither mentioned nor illustrated.

The book is beautifully printed on fine paper. Anyone acquainted with the cost of color illustrations, particularly those printed in eight colors, will recognize how moderate is the price of \$12. As a matter of fact, every reader should be aware of his debt not only to the authors, but also to the Commonwealth Fund for having shared so large a percentage of the expense. The atlas probably represents the last major publication of work in which the late Dr. Blackfan participated. It could be considered a worthy memorial.

**Secretory Mechanism of the Digestive Glands.** By *B. P. Babkin*, M.D., D.Sc., LL.D., F.R.S.C., Research Professor of Physiology, McGill University, Montreal, Can.; Formerly Professor of Physiology in the University of Odessa, Russia, and in Dalhousie University, Halifax, Nova Scotia, Can. With 220 illustrations. Paul B. Hoeber, Inc., New York, N. Y. Price \$12.75. Cloth with 900 pages.

Dr. Babkin has brought out a book on the secretory mechanism of the digestive glands based upon several courses of lectures given by him during the last ten years to postgraduate students at McGill University and some lecture-reviews presented at University College, London, and at the Universities of Edinburgh and Birmingham. Much of the material for this book is derived from work performed by him and his co-workers. There is a great deal of discussion of the secretion of the gastric juice and of the gastric mucus. In addition there is a detailed discussion of the secretion of the saliva, a subject which has not often been treated in such detail in many texts.

The book is well illustrated and contains a huge bibliography. It is an excellent review for those men interested in the mechanism of the secretion of the digestive glands and will be of particular value to research laboratories. In view of the marked revival of interest in both theoretical and clinical gastroenterology during the last few years, it should prove useful to the gastroenterologist doing research and teaching, but the book will have little value to the average clinician.

C. J. B.

**An outline of Tropical Medicine.** By *Otto Saphir*, M.D., Director of the Department of Pathology of the Michael Reese Hospital; Professor of Pathology, University of Illinois College of Medicine. Michael Reese Foundation, Chicago, Ill. Cloth with 86 pages.

**An Introduction to Public Health.** By *Harry Stoll Mustard*, Director DeLamar Institute of Public Health, College of Physicians and Surgeons, Columbia University. Second edition. The Macmillan Co., New York, N. Y. Price \$3.25. Cloth with 283 pages.

**Internes' Handbook.** By *M. S. Dooley*, M.D., A.B., Professor of Pharmacology, and *Maynard E. Holmes*, M.D., F.A.C.P., Professor of Clinical Medicine, Third edition. J. B. Lippincott Company, Philadelphia, Pa. Price \$3.00. University of Syracuse Medical School. Cloth with 579 pages.

## ISOIMMUNITY TO THE RH FACTOR AS A CAUSE OF BLOOD TRANSFUSION REACTIONS

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IN 1939 Levine and Stetson<sup>1</sup> reported the case of a woman who, after delivering a macerated fetus, developed a hemolytic reaction to a transfusion of blood from her husband. The blood of the husband and wife belonged to group O, but the recipient's serum was shown to contain an agglutinin which acted against the cells of about 80 per cent of persons belonging to Group O. The agglutinable factor was not M, N, or P. The potency of the agglutinin was diminished in two months and had disappeared from the blood in one year. It was concluded that the antibody was an isoimmune phenomenon which might have resulted from disintegration products from the dead fetus. Injections of agglutinable blood cells into rabbits failed to yield an antiserum. Landsteiner and Wiener,<sup>2</sup> in 1940, using agglutinin sera prepared by injecting rabbits with erythrocytes of rhesus monkeys, demonstrated antibodies exhibiting specificity apparently similar to the serum described by Levine and Stetson. The rabbit serum agglutinated the cells from approximately 85 per cent of human beings. They designated the agglutinable factor by the initials Rh. In the same year, Wiener and Peters<sup>3</sup> reported three cases in which repeated transfusions of Rh-positive blood into Rh-negative recipients stimulated the formation of anti-Rh agglutinins resulting in hemolytic reactions. Levine and co-workers<sup>4</sup> demonstrated the probability that erythroblastosis fetalis is caused by the development of antibodies in the Rh-negative mother against the Rh-positive blood of the fetus, resulting in a hemolytic type of anemia in the infant. They also described transfusion reactions in the mother from the presence of anti-Rh antibodies developed by pregnancy.<sup>5</sup>

Some individuals whose blood is Rh negative would seem to possess the potentiality of developing anti-Rh agglutinins by either of two mechanisms: by receiving blood in repeated transfusions from Rh-positive donors or by bearing children whose blood is Rh positive. Since approximately 15 per cent of persons of the white race in the United States have blood which is Rh negative, it might be considered that the development of isoimmunity to the Rh factor would be a common phenomenon through either of the two mechanisms. Actually, although the combination of Rh-positive child and Rh-negative mother occurs in about 1 of 10 pregnancies, only 1 pregnancy in 400 results in hemolytic disease of the child. It has also been noted that not all Rh-negative recipients are immunized by repeated transfusions of Rh-positive blood.

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It is particularly desirable to obtain some perspective on the incidence of isoimmunity in a general transfusion service because the prevention of transfusion reactions due to the anti-Rh agglutinin requires special laboratory tests preliminary to transfusion. The military employment of multiple transfusions of Group O donors to recipients of any blood group without preliminary cross-matching also raises the question of possible mortality from isoimmunization to the Rh factor.

#### METHODS OF INVESTIGATION

During a period of eighteen months (March 1, 1943, to Sept. 1, 1944) a series of 5,386 consecutive blood transfusions given to patients in the State University of Iowa Hospitals, under the supervision of staff members of the Blood Transfusion Service, was studied. The majority of the recipients were chronically ill. Transfusions were given without regard to the Rh type of the donor or the recipient. Many recipients received multiple transfusions. The obstetric history of female patients was disregarded, at least before transfusion. The recipients included both adults and children who were patients in a general hospital of approximately 800 beds. The blood of both donor and recipient was grouped with anti-A and anti-B sera of high titer, using the centrifuge technique. Most of the transfusions involved blood of homologous group. Preliminary cross-matching was performed at room temperature, the erythrocyte-serum suspensions being centrifuged and read within three minutes. In the great majority of cases, blood preserved in the modified Rous-Turner mixture<sup>6</sup> and stored from one to thirty days at from 4 to 6° C. was employed, although some transfusions of citrated blood, both fresh and stored, were given.

Most of the recipients were visited after transfusion by a member of the staff of the Blood Transfusion Service. Chills, fever, urticaria, dyspnea and other signs of reactions were noted. The post-transfusion urine was examined for hemoglobin. In cases of reaction associated with blood transfusion, the blood of the donor and recipient was typed for the Rh factor and the blood was cross-matched to determine the presence of anti-Rh agglutinins. These tests were performed by incubating a mixture of appropriate erythrocytes and serum for sixty minutes at 37° C., resuspending, and centrifuging for one minute. Then, after gentle shaking, the suspension was examined in the microscope for the presence of even small clumps.

#### CLASSIFICATION AND INCIDENCE OF REACTIONS

Reactions were classified according to the chief clinical manifestation. Many were characterized by chills and fever of only a few hours' duration. In these, the presence of hemoglobinemia was excluded by examination of the recipient's serum for hemoglobin and bilirubin. Urticarial reactions were easy to identify. Hemolytic reactions were diagnosed by the presence of hemoglobin in the post-transfusion urine or by the demonstration of free hemoglobin or increased bilirubin in the recipient's serum. Jaundice was determined by inspection. Gastrointestinal reactions were characterized by nausea and vomiting; their nature is uncertain. Cardiovascular reactions were evident by the clinical signs of left-sided cardiac embarrassment: dyspnea, cyanosis, coarse râles in the lungs, and prompt cessation after the application of tourniquets to the extremities.

The incidence of reactions due to all causes was as follows:

Transfusions without reactions	5,200
Transfusions with reactions of all types	186 (3.4%)
Total transfusions	5,386

The 186 transfusion reactions were classified as follows:

Chills and fever	101 (1.8%)
Urticaria	44 (0.8%)
Hemolysis	12 (0.2%)
Jaundice	8 (0.1%)
Gastrointestinal	11 (0.2%)
Cardiovascular	10 (0.1%)

In the 186 reactions of all types, but six were found which could be attributed to isoimmunity to the Rh agglutinin. This constitutes an incidence of reactions due to the Rh factor of 0.1 per cent in the 5,386 blood transfusions. Isoimmunity was responsible for 3.2 per cent of the 186 reactions in the series. Sensitization to the Rh factor was attributed to multiple transfusions in four instances; pregnancies were responsible in the other two. There was one fatality.

TABLE I

BLOOD TRANSFUSION REACTIONS ANALYZED ACCORDING TO THE NUMBER PER RECIPIENT AND SEX OF THE RECIPIENT

NUMBER OF TRANSFUSIONS PER RECIPIENT	MALE RECIPIENTS			FEMALE RECIPIENTS		
	NUMBER OF RECIPIENTS	TOTAL TRANSFU- SIONS	NUMBER OF REACTIONS	NUMBER OF RECIPIENTS	TOTAL TRANSFU- SIONS	NUMBER OF REACTIONS
1	508	508	15	532	532	17
2	205	410	17	194	388	15*†
3	157	471	9	121	363	9‡
4	77	308	8§	61	244	15
5	53	265	6	32	160	9
6	32	192	4	19	114	7
7	24	168	4	13	91	1
8	10	80	1	10	80	6
9	10	90	3	1	9	1
10	7	70	3	5	50	1
11	4	44	0	5	55	0
12	3	36	2¶	4	48	4
13	4	52	1			
14	3	42	2	1	14	2
15	1	15	0			
16	3	48	10**	2	32	1
17	1	17	0	2	34	0
19	1	19	1	1	19	0
20	1	20	0			0
21	1	21	0	1	21	0
23	2	46	7			
24	1	24	1			
25	1	25	0			
28	1	28	0			
40	1	40	0			
93	1	93	4			
Total	1,112	3,132	98 (3.1 ± 0.2%)	1,004	2,254	88 (3.9 ± 0.4%)
Total number of transfusions				5,386		
Total number of reactions				186 (3.4 ± 0.2%)		

\*Includes Case 44-1090, patient sensitized by pregnancies.

†Includes Case 44-0535, patient sensitized by transfusions not included in this series.

‡Includes Case 33-10386, patient probably sensitized by single pregnancy.

§Includes Case 43-11891, patient sensitized by transfusions.

||Includes Case 33-42221, patient sensitized by transfusions.

¶Includes Case 38-15931, patient sensitized by transfusions.

\*\*Includes seven urticarial reactions in one recipient.

In Table I are recorded the data on transfusion reactions distributed according to the sex of the recipients and the number of transfusions received by each. In any large group of recipients receiving multiple transfusions, the incidence of reactions due to isoimmunity to the Rh factor might be expected to be higher in females than in males because many of the former have had the added factor of pregnancy. There appears to be a statistically significant difference in the incidence of transfusion reactions of all types in the males and females of this series ( $3.1 \pm 0.2$  per cent as opposed to  $3.9 \pm 0.4$  per cent), although two males and two females were actually shown to have been sensitized by previous transfusions. With improved serologic methods, a greater number of reactions might be proved to be due to isoimmunity to the Rh factor.

Analysis of the data sheds some light on the probability of the Rh-negative recipient becoming sensitized to the Rh factor by receiving multiple blood transfusions. No patient in this series developed isoimmunity with less than four transfusions. Taking four blood transfusions as a purely arbitrary minimum for purposes of calculation, there were 242 males and 157 females who received four or more transfusions. It can be assumed that approximately 15 per cent of the 399 recipients, or sixty persons, were Rh negative. Only four of these were proved to have become immunized to the Rh factor, an incidence of 6.6 per cent.

#### CASE HISTORIES OF PATIENTS SHOWING ISOIMMUNITY TO THE RH FACTOR

CASE 43-11894.—Man, physician, aged 37 years, Group O. A gastrectomy was performed for a duodenal ulcer. The patient received transfusions of homologous group blood Oct. 28 and Oct. 30, 1943, without reaction. Nov. 27, 1943, he received another transfusion which was accompanied by urticaria. A fourth transfusion Jan. 25, 1944, was associated with a chill and fever of  $103^{\circ}$  F. There were no further signs or symptoms. The blood of the last donor was found to be Rh positive. The recipient's blood was Rh negative, but no anti-Rh agglutinin could be demonstrated in the serum. Isoimmunity in this instance was attributed to repeated transfusions.

CASE 38-15984.—Man, aged 21 years, Group O. The patient was under treatment for tuberculosis of the left hip. In a period of a month, from Oct. 10, 1943, to Nov. 11, 1943, he received ten blood transfusions of homologous group. The first four transfusions were without incident. There were chills and fever with the fifth and sixth. No reaction followed the seventh and eighth. Violent chills and fever were associated with the ninth and tenth transfusions. The recipient's cells were Rh negative and the serum contained an anti-Rh agglutinin. The cells of the last two donors were Rh positive. The cells of the father of the patient were found to be Rh negative. Subsequently the son was given two transfusions of the father's blood without reaction.

CASE 38-42221.—Woman, aged 37 years, Group A. After an incomplete abortion, the patient received blood transfusions July 6, 7, and 11, 1944, without reactions. Aug. 1, 1944, another transfusion of homologous blood group was accompanied by violent chills, pain in the thighs, and temperature of  $101.2^{\circ}$  F. A small amount of free hemoglobin was present in the blood serum after transfusion and there was hemoglobinuria. The recipient's blood was demonstrated to be Rh negative and contained agglutinins against the Rh-positive blood of the last donor. Aug. 20, 1944, she received another transfusion of Rh-negative blood without reaction. The obstetric history was interesting. There had been nine pregnancies, all deliveries having been performed in the University Hospitals. The sixth pregnancy had resulted in a premature infant who died in the hospital without signs of erythroblastosis fetalis. The blood of seven living children was typed for the Rh factor. The children from the first, second, and fourth pregnancies were Rh positive; those from the third, fifth, seventh, and eighth

were all Rh negative. This was concluded to be another example of isoimmunity developing from repeated transfusions, although the occurrence of the abortion suggests a possible trans-placental mechanism.

CASE 44-1090.—Woman, aged 49 years, Group B. During treatment for metrorrhagia from chronic cervicitis, the patient received a blood transfusion of homologous group Feb. 2, 1944. This was associated with chills and fever. The next day she was given a second transfusion which resulted in a violent chill, fever of 103.8° F., and transient jaundice which appeared in about six hours. The blood cells were found to be Rh negative and the serum contained anti-Rh agglutinins which strongly agglutinated the cells of many Rh-positive bloods by the centrifuge technique without incubation. The blood from the last donor was Rh positive. Three transfusions of Rh-negative blood were later given without reaction. The children from the first three pregnancies were living. In subsequent pregnancies, there had been three miscarriages, one stillbirth, and one child who died of umbilical hemorrhage two weeks after birth. It is probable that isoimmunity in this instance was due to repeated pregnancies, although the last had occurred ten years before.

CASE 38-10586.—Woman, aged 23 years, Group A. The patient was a primipara who entered the hospital with signs of toxemia of pregnancy. She had previously received a blood transfusion of homologous group Sept. 23, 1943, without reaction. Aug. 3, 1944, a second transfusion was given. When 350 c.c. of blood had been injected, the recipient had a chill, the temperature rose, and severe dyspnea developed. Coarse râles were present in the lungs. The transfusion was immediately discontinued and a specimen of the recipient's blood was promptly collected. The serum was yellow when contrasted with the normal color of the pre-transfusion specimen. There was no free hemoglobin in the serum, but the van den Bergh reaction was 6.1 units, delayed direct. The urine for the next twenty-four hours contained no hemoglobin but was colored a deep brown with pigment which gave the chemical reactions for bilirubin. The transfusion reaction precipitated labor and the patient was delivered of a dead infant estimated to be eight months old. The child was somewhat jaundiced and the abdomen was distended with ascitic fluid. Blood taken from the right ventricle yielded a yellow serum with a van den Bergh reaction of 4.0 units, delayed direct. The baby's erythrocytes contained abnormally high numbers of normoblasts and megalocytes. The post-mortem findings were consistent with a diagnosis of erythroblastosis fetalis. The mother's blood cells were Rh negative and the infant's were Rh positive. The mother's serum contained no agglutinins acting against the baby's cells or against the Rh-positive blood of the donor. No antibody could be demonstrated by Wiener's "blocking test" where the mother's serum and the baby's cells were incubated and anti-Rh serum then added. Agglutination was not inhibited by this procedure. Fourteen days after the transfusion, the mother's serum contained a weak, but definite, agglutinin against Rh-positive cells. This case is notable for several features. It is unusual for erythroblastosis to occur during the first pregnancy. The development of isoimmunity from the first transfusion is also unlikely. A transfusion reaction occurred, apparently from incompatibility to the Rh factor, although no agglutinin could be demonstrated. Wiener's blocking test failed to reveal the presence of an antibody. An anti-Rh agglutinin appeared in the serum within fourteen days after the transfusion and delivery. The free hemoglobin from the intravascular hemolysis was apparently so rapidly converted to bilirubin that none was detected in the serum immediately after the transfusion. A considerable amount of a pigment, apparently bilirubin, was excreted in the urine for many hours after hemolysis.

CASE 44-6538.—Woman, aged 30 years, Group B. The patient had never been pregnant. In 1933 she developed bronchiectasis and lobectomy was performed in the University Hospitals. The operation was complicated by empyema thoracis. During a stormy postoperative course, she received three blood transfusions (Group B) Oct. 17, 18, and 19, 1933. Each was followed by fever, but it was impossible to determine whether this was due to the infection or to the transfusions. It was the judgment of those in attendance that there had been no transfusion reactions. After operation for a gangrenous appendix, the patient received another transfusion Dec. 23, 1934, without reaction. A diaphragmatic abscess developed and was drained. Another transfusion was given without reaction Dec. 28, 1934.

During the intervening years an abscess of the lung developed at the site of the lobectomy and the patient was readmitted to the hospital with hemoptysis. On July 7, 1944, she received a transfusion of Group B blood without reaction. Another transfusion was given July 19, 1944. The patient was carefully observed during the administration of the 500 c.c. of blood because a new type of apparatus was being used. No untoward symptoms were noted during transfusion, but about twenty minutes after conclusion of the procedure, the patient had a chill and the temperature rose to 102° F. She was not considered to be seriously ill by the physician in attendance and it was not until fourteen hours later that a small amount of urine was voided which contained hemoglobin. The patient appeared apathetic and the skin was flushed. The heart rate was fast; the blood pressure measured 60 mm. of mercury, systolic, and the diastolic pressure could not be measured. Since there had been no recent loss of blood and there was vasodilatation, it was considered that the condition was primary shock. The blood pressure was restored and maintained at normal levels with subcutaneous injections of ephedrine during the day. The daily urine volume varied from 50 c.c. to 250 c.c. for the remainder of life. The values for urea nitrogen and creatinine of the blood increased rapidly. Solutions of dextrose, saline, and sodium lactate were administered parenterally. The renal pelvises were lavaged with warm water through the cystoscope. With continuing oliguria, decapsulation of the kidneys was performed by Dr. R. H. Flocks, of the Department of Urology, July 22, 1944. The kidneys were normal in size and the capsules stripped with ease. The operator thought that the kidneys were slightly edematous. The urinary excretion increased but little with any type of therapy. The patient became stuporous and died July 31, 1944, on the eleventh day after transfusion. The blood urea nitrogen at the time of death was 87.5 mg. per 100 c.c., and the creatinine of the blood was 14.5 mg. per cent.

Post-mortem examination was not permitted. A blood specimen taken from the patient fourteen hours after transfusion was examined. The serum was pink and spectrophotometric tests showed the pigment to be hemoglobin. No methemoglobin was present in the serum. The urine voided during the fourteen hours after transfusion exhibited in the spectrophotometer the typical bands of hemoglobin as well as those of methemoglobin. The patient's cells were Rh negative and those of the last donor were Rh positive. The recipient's serum contained an agglutinin which reacted against the donor's cells, after incubation for sixty minutes at 37° C., but did not react when the centrifuge technique without incubation was employed.

#### CLINICAL MANIFESTATIONS OF TRANSFUSION REACTIONS DUE TO ISOIMMUNITY

From the analysis of transfusion reactions and the case histories of patients showing isoimmunity, it is evident that transfusion reactions due to sensitivity to the Rh factor cannot be differentiated by clinical symptoms and signs from those due to other causes. In some instances, the chills and fever, resulting from the transfusion of Rh-incompatible blood, are clinically indistinguishable in severity or in course from the symptoms resulting from the injection of pyrogens of bacterial origin. There is no conclusive evidence from this study that urticarial reactions are related to isoimmunity to the Rh factor.

Clinically there seem to be slight differences between the hemolytic reactions due to the Rh factor and those caused by transfusion of blood incompatible in the ABO system. These differences are probably quantitative rather than qualitative. They are explained by the relative weakness of the anti-Rh agglutinins. The recipient who hemolyzes blood by means of an anti-Rh antibody frequently does so at a slower rate, and the total circulating free hemoglobin is not great at any one moment. The symptoms are thus slow in developing, as witness the fact that the patient who suffered the fatal reaction exhibited no signs of clinical significance until after the entire transfusion had

been concluded. From these considerations it is probable that some instances of isoimmune reactions escaped notice in this series because of complete lack of clinical symptoms. These could only be detected by laboratory procedures which would reveal failure to attain the expected blood count after transfusion or by the demonstration of increases in serum bilirubin level after transfusion. Such indications of increased rate of hemolysis, of course, are not specific for the anti-Rh antibody but would apply equally well to the increased loss of blood which had not been properly preserved.

#### DIFFICULTIES IN TESTING FOR RH INCOMPATIBILITIES

There are many obstacles which present difficulty in the search for incompatibilities to the Rh factor in a blood transfusion service. The first is the lack of adequate sources of Rh-typing sera. Thus far, antisera derived from laboratory animals have not proved satisfactory. Serum collected from the rare patient who develops sufficient agglutinin titer, either by repeated transfusions or by pregnancy, constitutes the only source. The problem is still further complicated by the demonstration by Wiener<sup>3</sup> of at least three antisera which are required to determine with certainty that cells from any source are Rh negative.

There is a disturbing lack of adequate methods of demonstrating anti-Rh antibodies in the laboratory. Although hemolysis of Rh-positive blood frequently, or perhaps always, occurs when it is transfused into a recipient possessing an anti-Rh antibody, no one has been able to demonstrate hemolysis in the test tube. Where isoimmunity to the Rh factor probably exists, no agglutinin can be demonstrated in many instances by the methods now in use.

It is frequently necessary to work with weak anti-Rh agglutinins in cross-matching blood. Difficulty is occasionally encountered in determining whether significant agglutination has occurred. This is particularly true in dealing with preserved blood. During storage the erythrocytes sediment and sometimes adhere so that some agitation of the suspension is necessary to separate them. On the other hand, it requires little shaking to break up the agglutination caused by the anti-Rh serum. The interpretation of the finding of small clumps of cells in the erythrocyte-serum suspension is difficult. The differentiation between rouleau formation and true agglutination offers difficulties which are aggravated when preserved blood is involved. The erythrocytes during storage frequently lose their biconcave, discoid shapes so that when rouleaux form, they appear as clumps rather than as stacks of coins. The usual method of distinguishing these from clumps due to the presence of the anti-A or anti-B agglutinins is to dilute the cell-serum suspension with saline and agitate, the rouleaux being broken up readily by this procedure. This maneuver, however, also breaks up the clumps formed by the anti-Rh serum, especially when the titer is weak.

The time required for incubation of cell-serum suspensions for the demonstration of the anti-Rh agglutinin (from thirty to sixty minutes) imposes a serious delay if the transfusion is required as emergency treatment. In view of the incidence of isoimmunity to the Rh factor presented in this paper, it is the practice of staff members of the Blood Transfusion Service to forego the

incubation of cell suspensions preliminary to urgent transfusions. The delay is considered more dangerous than the possibility of a reaction from Rh-incompatible blood.

#### SUMMARY AND CONCLUSIONS

1. A series of 5,386 consecutive blood transfusions was studied. Multiple transfusions were given to recipients without regard to Rh type or to obstetric history of female recipients. Transfusion reactions of all types were studied for evidence of isoimmunization with the Rh factor.

2. The incidence of transfusion reactions of all types in the series was  $3.4 \pm 0.2$  per cent. In 3,132 transfusions given to 1,112 males, the incidence of reactions was  $3.1 \pm 0.2$  per cent. In 1,004 female recipients of all ages, the incidence of reactions was  $3.9 \pm 0.4$  per cent in 2,254 transfusions. This is considered to be a statistically significant difference in the sexes which may possibly be explained by isoimmunity to the Rh factor developed during pregnancy.

3. In the 186 transfusion reactions of all types, only six were found which could be attributed to isoimmunity to the Rh factor, an incidence of 0.1 per cent in the 5,386 transfusions. Isoimmunity was attributed to multiple transfusions in four instances and to pregnancy in two. There was one fatality from transfusion of Rh-positive blood into a recipient who had been sensitized by multiple transfusions.

4. There were 1,717 recipients receiving from one to three transfusions. In this group, none was sufficiently sensitized to the Rh factor to give clinically significant reactions. It is estimated that of the 399 recipients receiving four or more transfusions, approximately sixty were Rh negative. Only four, or 6.6 per cent, of these were immunized to the Rh factor by multiple blood transfusions.

5. The case histories of the six recipients with isoimmune reactions are reported. Preliminary cross-matching for the anti-Rh agglutinin would not have prevented all of the reactions. A series of increasingly severe reactions in a recipient cannot be depended upon as adequate warning of developing sensitivity to the Rh factor.

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## STUDIES OF SICKLE-CELL FORMATION IN NORMAL SALINE, PLASMA, AND SERA WITH CARBONIC ANHYDRASE INHIBITORS

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TO DATE, no reports have been made upon the presence, or absence, of the enzyme carbonic anhydrase in relation to the phenomenon of sickling erythrocytes. Also, considerable discrepancy exists among various reports<sup>1-4</sup> concerning the ability of susceptible erythrocytes to sickle when washed with normal saline and resuspended in normal saline, plasma, or sera. We desire to report controlled studies upon sickle-cell formation under the above circumstances, studies on the role of carbonic anhydrase in sickle-cell formation, and the effect of its inhibition.

### MATERIAL

The blood for these studies was obtained from British West Indian or Panamanian Negroes admitted to this hospital for a variety of complaints not related to the sickling phenomenon. Blood from one outpatient with active sickle-cell anemia in an asymptomatic period was also used. Sickle-cell preparations were made in all cases by using No. 1 cover slips and sealing the edges with petrolatum. The preparations were checked immediately and in twenty-four hours. All blood used showed at least 80 per cent sickled forms in twenty-four hours at room temperature (28° C.).

### I. EFFECT OF NORMAL SALINE, PLASMA, AND SERA SUSPENSION MEDIUMS ON SICKLE-CELL FORMATION

*Material and Procedure*—Blood from fifteen patients with sickle anemia and one with active sickle-cell anemia was available for this study. The blood specimens were, where desired, defibrinated in the usual manner and separated into cells and sera by centrifuging and resuspending the cells in normal saline (0.85 per cent). For other uses 10 c.c. of blood were placed directly into a 30 c.c. volume test tube containing 17 mg. (+) of potassium oxalate. (A one-half saturated solution of potassium oxalate was used; 0.1 c.c. of this solution was placed in the bottom of each tube and evaporated. By analysis, this has been found to leave 17 mg. [±] of potassium oxalate in each tube.) The oxalated blood was centrifuged and the plasma removed; then the packed erythrocytes were washed in the following manner. Conical 15 c.c. centrifuge tubes were used and 0.5 c.c. of packed erythrocytes and 14 c.c. of normal saline were thoroughly mixed and then centrifuged for four minutes at 2,000 r.p.m.; the supernatant saline was poured off and the packed erythrocytes were resuspended in saline and the process repeated the number of times indicated. Following the last centrifugation the supernatant saline was removed and the volume of packed erythrocytes was made up to 1 c.c. with either normal saline, plasma, or serum as desired. From these suspensions, petrolatum-sealed cover-slip prepara-



tions were made and examined immediately and in twenty-four hours. The blood from the patient with active sickle-cell anemia was the only one which showed sickled forms (15 per cent) immediately after the sealed preparation was made.

TABLE I

PERCENTAGE SICKLING IN TWENTY-FOUR HOURS WHEN WASHED SUSCEPTIBLE ERYTHROCYTES ARE SUSPENDED IN SALINE, SAME, AND OTHER PLASMA AND SERA

TIMES WASHED	SUSPENDED IN	CASE															
		1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
5	Normal saline	100	90	80	90	95	80	80	95	90	95	100	90	100	95	100	90
10	Normal saline	-	-	-	-	-	-	20	30	50	-	25	20	30	35	40	20
20	Normal saline	-	-	-	-	-	-	-	-	-	-	Neg	Neg	5	Neg	Neg	Neg
5	Same plasma							80	90	95	-	100	90	100	95	95	90
5	Same serum							80	95	95	-	95	85	95	95	95	85
10	Same plasma							30	40	30	-	35	25	40	35	40	30
10	Same serum							25	35	35	-	40	25	35	40	35	35
5	Other plasma							75	95	90	-	90	90	95	90	95	95
5	Other sera							70	90	90	-	95	85	90	85	95	90
10	Other plasma							20	20	25	-	30	20	30	30	20	15
10	Other sera							30	15	20	-	30	10	25	10	25	20
Control	untreated blood	100	90	80	90	90	80	85	100	100	90	100	90	100	100	100	90

\*Active sickle-cell anemia.

*Results.*—From Table I it is seen that washing susceptible erythrocytes five times with normal saline has no effect upon their ability to sickle in twenty-four hours when they are resuspended in normal saline. Washing the erythrocytes ten times reduces their ability to sickle from 50 to 75 per cent, while washing twenty-times almost always removes their ability to assume sickled forms in normal saline.

In the experiments using the patients' own plasma and serum for suspending the cells after washings, five washings had little or no effect in preventing sickle formation, while washing ten times reduced the ability of the cells to assume sickled shapes from 60 to 70 per cent.

In experiments using plasma and sera from normal white and Negro persons of the same blood groups as the cases of sicklemia being studied, washing the erythrocytes five times before suspending in the plasma or sera had little or no effect, while washing ten times reduced the ability of the cells to assume sickled shapes from 45 to 75 per cent. The implications of these findings will be discussed later.

## II. EXPERIMENTS ON OXYGENATED BLOOD WITH SUBSTANCES KNOWN TO BE CARBONIC ANHYDRASE INHIBITORS

*Material and Procedure.*—Carbonic anhydrase, an enzyme which catalyzes both phases of the reversible reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$ , is present in all mammalian erythrocytes. Its presence accelerates the reaction above so that the exchange of gases can occur under normal conditions during the passage of blood through the pulmonary circulation. No studies have been reported upon

TABLE II  
THE EFFECT OF CARBONIC ANHYDRASE-INHIBITING COMPOUNDS ON SICKLE-CELL FORMATION IN OXYGENATED BLOOD

	EXAMINED	CASE													
		1*	6	7	8	9	10	11	12	13	14	15	16		
Sulfanilamide (1:1,000)	Immediately	-	Neg.	Neg.	-	Neg.	Neg.	-	-	-	-	Neg.	Neg.		
Ammonium thiocyanate (1:1,000)	In 24 hours	-	40†	55	-	100	60	-	-	-	-	35	40		
	Immediately	-	-	-	-	Neg.	-	Neg.	Neg.	-	-	Neg.	-		
Zinc acetate (1:1,000)	In 24 hours	-	-	-	-	100	20	40	80	-	-	40	-		
	Immediately	12	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.		
Sodium cyanide (1:1,000)	In 24 hours	12	1	Neg.	1	1	1	Neg.	Neg.	1	Neg.	Neg.	Neg.		
	Immediately	10	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.		
Sodium acetate (1:1,000)	In 24 hours	10	Neg.	Neg.	Neg.	1	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.		
	Immediately	11	-	Neg.	-	-	-	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.		
Zinc sulfate (1:1,000)	In 24 hours	100	-	90	-	-	-	100	90	100	95	100	90		
	Immediately	10	-	Neg.	-	-	-	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.		
	In 24 hours.	10	-	Neg.	-	-	-	1	4	Neg.	1	Neg.	Neg.		

\*Active sickle-cell anemia.

†Percentage sickled forms.

the presence of, or the relation between, carbonic anhydrase and the sickling of erythrocytes.

Sulfanilamide and zinc salts,<sup>5</sup> ammonium thiocyanate,<sup>6</sup> and cyanide compounds<sup>7</sup> have been shown to inhibit or destroy the action of carbonic anhydrase, and these substances were tested against the blood of sicklemic patients and of one with active sickle-cell anemia. The oxalated bloods were thoroughly oxygenated and normal saline solutions of the various test compounds were added to make final 1:1,000 dilutions in the blood. They were thoroughly mixed, then petrolatum-sealed cover-slip preparations were made immediately. These preparations were examined at once, in twenty-four hours, and in forty-eight hours. The twenty-four and forty-eight hour results were similar and only the twenty-four hour figures are given.

*Results.*—In Table II are the figures representing the percentage of sickled forms present. Sulfanilamide and ammonium thiocyanate were inconstant and incomplete in their effects. Zinc salts and the cyanide radical were complete in their effect, preventing the formation of sickle forms beyond the numbers already present, as in Case 1 of active sickle-cell anemia. The sickled forms in this case could not be changed to discoid forms by passing pure oxygen over the blood for ten minutes before starting the experiments. Sodium acetate was used as a control since the zinc salt used was an acetate and the cyanide radical was combined with sodium. It showed no inhibition of the sickling phenomenon.

### III. EXPERIMENTS ON CARBONATED BLOOD WITH SUBSTANCES KNOWN TO BE CARBONIC ANHYDRASE INHIBITORS

Blood was exposed to a pure carbon dioxide stream for fifteen minutes, then mixed with carbonic anhydrase-inhibiting agents (zinc acetate, sodium cyanide, 1:1,000), and sealed cover-slip preparations were examined immediately and in twenty-four hours. Sickling was complete when preparations were examined at these times. The cover slips were then pried off and air was admitted. In contrast to the usual preparations treated by removing the cover slips after sickling has occurred, there was no resumption of the normal discoid forms.

Likewise, fully oxygenated blood was mixed with the same inhibiting agents and cover-slip preparations were made. No sickling was present immediately, in twenty-four, or in forty-eight hours.

### DISCUSSION

Murphy and Shapiro<sup>8</sup> studied one case of active sickle-cell anemia and felt that sickle-cell formation in normal saline suspensions was variable and was influenced by the erythrocyte concentrations. They felt that high erythrocyte concentrations metabolized the oxygen in the saline and resulted in sickling, while low erythrocyte suspensions, failing to metabolize the oxygen, did not show sickle forms. They do not indicate the number or extent of washings with which the cells were treated, and no comparisons can be drawn between their work and that reported here. Our studies were completed when their report became available and we have not attempted to check the effect of cell concentrations; however, in our experiments the cell concentrations ran as high or higher than those present in the unaltered blood from our patients.

A consideration of the percentages of sickle-cell formation in saline, plasma, and sera using saline-washed cells immediately leads to the opinion that long washings remove some substance necessary for sickling, or for the exchange of carbon dioxide and oxygen which makes sickling possible. This substance is not contained in plasma or serum, inasmuch as susceptible erythrocytes after twenty washings with normal saline do not assume sickle forms when resuspended in their original plasma or sera. The substance might be carbonic anhydrase. No determinations of carbonic anhydrase content were made on the saline washing fluid because of technical difficulties. However, Scott and Mendive<sup>9</sup> record a 29 per cent loss of carbonic anhydrase activity by washing erythrocytes three times with 0.9 per cent sodium chloride and then extracting with alcohol and chloroform.

From the experiments on carbonic anhydrase inhibition we feel that the evidence indicates that carbonic anhydrase is probably present in normal, active amounts in the erythrocytes of people with the sickling phenomenon and does not enter into the phenomenon of sickling beyond its normal function, namely, facilitating the exchange of carbon dioxide and oxygen. Inasmuch as sulfanilamide and ammonium thiocyanate gave incomplete and inconstant inhibition of the carbonic anhydrase activity, it is possible that some other mechanism may be involved.

#### SUMMARY

1. Erythrocytes capable of assuming sickled shapes will do so when resuspended in normal saline, their original plasma or serum, or in other compatible plasma or serum, providing they have not been washed more than five times in normal saline.

2. Multiple washings of the erythrocytes remove some substance, possibly carbonic anhydrase, which is necessary for the phenomenon of sickling.

3. Zinc acetate or sodium cyanide in concentrations of 1:1,000, when added to oxygenated blood, prohibits sickle-cell formation.

4. Zinc acetate or sodium cyanide in concentrations of 1:1,000, when added to carbonated blood, does not reverse the sickled forms and they do not resume discoid shapes when exposed to air or oxygen.

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## NONSPECIFIC COMPLEMENT-FIXING ANTIGEN IN EMBRYONIC EGG TISSUES

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COMPLEMENT-FIXING antigens prepared from embryonated chick eggs are being used extensively for the diagnosis of virus and rickettsial diseases. Bengtson<sup>1, 2</sup> prepared antigens for "Q" fever and murine typhus from infected yolk sac cultures, while McKee, Rake, and Shaffer<sup>3</sup> prepared antigens for lymphogranuloma venereum from the same tissue. Reynolds and Pollard<sup>4</sup> employed commercial typhus vaccine as a complement-fixing antigen for typhus fever. Plotz<sup>5</sup> recommended the use of purified rickettsial antigens for the diagnosis and differentiation of epidemic and murine typhus, and Smadel, Wertman, and Reagan<sup>6</sup> employed the same principle in the preparation of a psittacosis antigen. Yanamura and Meyer<sup>7</sup> prepared a psittacosis antigen from chick embryo cells grown on a semisolid medium used by Zinsser, Plotz, and Enders<sup>8</sup> for the cultivation of rickettsiae, while Plotz and Wertman<sup>9</sup> prepared a Rocky Mountain spotted fever rickettsial antigen from infected chick embryo cells grown on the same medium.

The fact that normal tissues contain antigen that reacts with Wassermann-positive sera is well known. Alcoholic extracts of infected and normal tissue have been described as Wassermann antigens by Detre<sup>10</sup> and Landsteiner, Müller, and Potzl.<sup>11</sup> Mazzini<sup>12</sup> described a microscopic flocculation test for syphilis in which the antigen was prepared from beef heart and egg yolk. This antigen contains an ether-insoluble, alcohol-soluble lipoid which reacts with the syphilitic antibody. Guggenheim<sup>13</sup> found that the injection of egg yolk caused the formation of antibodies of the Wassermann type in rabbits.

Since crude egg antigens are being used extensively in the complement fixation tests, it was thought advisable to study more thoroughly the nature of this nonspecific complement-fixing phenomenon.

In the several techniques advocated for the performance of complement fixation tests, the time of primary incubation and temperature has varied. Bronfenbrenner and Schlesinger<sup>15</sup> found that short periods of primary incubation at 37° C. were sufficient to permit the more active antigens and antibodies to unite and could be used for presumptive elimination of strongly positive sera. They stated that icebox incubation for from eight to ten hours, while increasing the degree of fixation, may give nonspecific reactions in that even traces of secondary antigens and their antibodies may cause fixation of complement. Kolmer, Rule, and Yagle<sup>14</sup> have similarly shown that a prolonged period of primary incubation at icebox temperature increases the fixation of complement.

Plotz<sup>5</sup> and Plotz and Wertman,<sup>9</sup> recognizing the possible danger of nonspecific reactions with secondary antigens at 8° C. overnight, yet wishing to use the more sensitive technique, advocated the use of highly purified antigens

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from which such nonspecific substances have been removed. With the use of such materials it has been found that the more sensitive technique of overnight fixation at from 4 to 8° C. yields much higher titers without interference of nonspecific reactions with syphilitic serum.

Because of the possibility that persons unfamiliar with the details of the preparation of these antigens might attempt to interchange the recommended techniques of primary incubation, it was decided to study the degree of nonspecific complement fixation with the use of normal egg antigens as well as with crude rickettsial antigens prepared from infected eggs when primary incubation of one hour at 37.5° C. and of eighteen hours at from 4 to 8° C. was employed.

*Methods.*—Complement titrations were carried out with varying quantities of a 1:30 dilution of commercial lyophilized guinea pig serum; that is, 0.08 c.c. to 0.26 c.c. with increments of 0.02 c.c. One exact unit was defined as the minimal amount of complement in which complete hemolysis of the sensitized sheep cells had occurred. A full unit was that amount in the second tube giving complete hemolysis or 0.02 c.c. greater than the exact unit. Two full units of complement in a volume of 0.5 c.c. were employed throughout the study. In order to establish whether any complement activity was lost, other than by specific fixation, parallel titrations of  $\frac{1}{2}$  unit, 1 unit,  $1\frac{1}{2}$  units, and 2 units were run for each period of primary incubation.

In the antigen titration, 0.25 c.c. of serum dilution, 0.25 c.c. of antigen dilution, and 0.5 c.c. of complement (containing 2 full units) were mixed for the primary incubation. After incubation, a suspension of sensitized red blood cells, which consisted of equal amounts of amboceptor (3) units and sheep erythrocytes (3 per cent), was added in 0.5 c.c. volume to each tube. The total volume in each tube was 1.5 c.c. Secondary incubation for thirty minutes at 37.5° C. was then employed. In reading the tests, a 4 plus (no hemolysis of the sensitized cells), and a 3 plus (25 per cent hemolysis of the cells) were taken as end points. A 2 plus (50 per cent hemolysis of the cells) or less was considered as not significant. Primary incubation, which was the only variable, was carried out for one hour at 37.5° C., four hours at from 4 to 8° C., and eighteen hours at from 4 to 8° C. The usual controls for the complement fixation technique were incorporated in each protocol.

Antigens were prepared from normal yolk sacs and embryos of developing hen eggs at the ages usually employed in preparing rickettsial and virus complement-fixing antigens; that is, 10- and 11-day-old developing chick embryos. The yolk sacs or embryos were shaken with glass beads and 10 per cent suspensions in saline were prepared. The suspensions were centrifuged at 1,500 r.p.m. for thirty minutes in an International Centrifuge, Type SB to remove the large particles, and the supernatant fluid from each suspension was employed as antigen.

The same pools of human sera from syphilitic and nonsyphilitic individuals were used throughout this study. The pool of sera from syphilitic persons was markedly positive with both Kahn and Kolmer techniques.\*

*Effect of Primary Incubation on Normal Egg Antigens.*—Various fractions of the prepared normal egg antigens were titrated against syphilitic and non-

\*Pooled serum from syphilitic and non-syphilitic individuals supplied by the Division of Serology, Army Medical School.

syphilitic sera. Twofold dilutions of antigen were tested at varying primary incubations against twofold dilutions of serum.

In the first test, normal 10 per cent yolk sac and normal 10 per cent embryo suspensions were studied (Table I). Since the nonsyphilitic sera tested at 1/5 and 1/10 dilutions were completely negative against all dilutions of antigen at the three periods of primary incubation, only the 1/5 dilution of these sera is included in the tables.

TABLE I  
EFFECT OF PRIMARY INCUBATION ON REACTION OF SYPHILITIC SERA AND NORMAL EGG ANTIGENS

SERUM	SERUM DILUTION	10 PER CENT NORMAL YOLK SAC SUSPENSION* TEST INCUBATED AT THE FOLLOWING			10 PER CENT NORMAL EMBRYO SUSPENSION* TEST INCUBATED AT THE FOLLOWING		
		1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.	1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.
Nonsyphilitic pool	1/5	0	0	0	0	0	0
Syphilitic pool	1/5	1/16	1/16	1/128	1/8	1/8	1/64
Syphilitic pool	1/10	1/8	1/8	1/64	1/8	1/8	1/32
Syphilitic pool	1/20	0	0	1/32	0	0	1/8
Syphilitic pool	1/40	0	0	1/16	0	0	1/4
Syphilitic pool	1/80	0	0	1/16	0	0	0

\*Twofold dilutions of antigen titrated.

In Table I it is demonstrated that both the normal yolk sac antigen and the embryo antigen are capable of fixing complement in the presence of syphilitic sera. It also illustrates that greater fixation results when the more sensitive technique of eighteen hours' incubation in the cold is employed.

It was then of interest to determine whether this nonspecific agent could be removed by ether extraction and further processing. The antigens were therefore extracted with ethyl ether, and the resulting emulsion and excess ether were discarded. The aqueous fractions were then titrated as before with the use of the same procedure (Table II).

TABLE II  
REACTION OF SYPHILITIC SERA AND ETHER-EXTRACTED NORMAL EGG ANTIGENS

SERUM	SERUM DILUTION	10 PER CENT NORMAL YOLK SAC, ETHER-EXTRACTED* TEST INCUBATED AT THE FOLLOWING			10 PER CENT NORMAL EMBRYO, ETHER-EXTRACTED* TEST INCUBATED AT THE FOLLOWING		
		1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.	1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.
Nonsyphilitic pool	1/5	0	0	0	0	0	0
Syphilitic pool	1/5	1/4	1/4	1/64	1/4	1/8	1/32
Syphilitic pool	1/10	<1/4†	<1/4	1/32	<1/4	1/4	1/16
Syphilitic pool	1/20	0	0	1/8	0	0	1/8
Syphilitic pool	1/40	0	0	1/4	0	0	0
Syphilitic pool	1/80	0	0	0	0	0	0

\*Twofold dilutions of antigen titrated.

†<1/4 indicates a 1 plus or 2 plus reaction at that dilution.

From the results shown in Table II, it is noted that while the ether extraction removed approximately one-half of the reacting antigen from normal yolk sac and normal embryo antigens, a considerable amount of antigenic material

still remained which was more active when the more sensitive technique of primary incubation for eighteen hours at from 4 to 8° C. was used.

Ether-extracted normal yolk sac suspensions and ether-extracted embryonic tissue were centrifuged in an angle centrifuge in the cold at 12,000 r.p.m. for one hour. The resulting sediment was resuspended to the original volume with 0.85 per cent saline solution and tested as was the supernatant fluid against the sera from syphilitic and nonsyphilitic individuals. In Table III it is shown that the antigen reacting with syphilitic sera remained in the supernatant fluid after centrifugation at 12,000 r.p.m. for one hour, while the sediments did not react even when the more sensitive technique was employed.

TABLE III  
REACTION OF SYPHILITIC SERA AND ETHER-EXTRACTED NORMAL EGG ANTIGENS

SERUM	SERUM DILUTION	SEDIMENT RESUSPENDED AND TESTED AT			SUPERNATANT TESTED AT		
		1 HR. AT	4 HR. AT	18 HR. AT	1 HR. AT	4 HR. AT	18 HR. AT
		37.5° C.	4 TO 8° C.	4 TO 8° C.	37.5° C.	4 TO 8° C.	4 TO 8° C.
<i>A. 10 Per Cent Normal Yolk Sac, Ether-Extracted, 12,000 r.p.m. for One Hour*</i>							
Nonsyphilitic pool	1/5	0	0	0	0	0	0
Syphilitic pool	1/5	0	0	0	1/4	1/4	1/64
Syphilitic pool	1/10	0	0	0	1/4	1/4	1/32
Syphilitic pool	1/20	0	0	0	0	1/2	1/8
Syphilitic pool	1/40	0	0	0	0	0	0
Syphilitic pool	1/80	0	0	0	0	0	0
<i>B. 10 Per Cent Normal Embryo, Ether-Extracted, 12,000 r.p.m. for One Hour*</i>							
Nonsyphilitic pool	1/5	0	0	0	0	0	0
Syphilitic pool	1/5	0	0	0	1/4	1/4	1/32
Syphilitic pool	1/10	0	0	0	1/4	1/4	1/16
Syphilitic pool	1/20	0	0	0	0	0	1/8
Syphilitic pool	1/40	0	0	0	0	0	1/4
Syphilitic pool	1/80	0	0	0	0	0	0

\*Twofold dilutions of antigen tested

*Removal of Syphilitic Antigen by Absolute Alcohol Extraction.*—Since Mazzini<sup>12</sup> has described a flocculating antigen prepared from absolute alcohol extracted beef heart and egg yolk for the diagnosis of syphilis, it seemed advisable to attempt the removal of the syphilitic-reacting substance from embryonic egg antigens by this method. A 10 per cent suspension of normal yolk sac that reacted with syphilitic sera was therefore extracted with absolute alcohol. The titration of the antigen before and after the treatment is given in Table IV.

TABLE IV  
10 PER CENT NORMAL YOLK SAC SUSPENSION—PRIMARY INCUBATION EIGHTEEN HOURS AT FROM 4 TO 8° C.

SERUM	DILUTION	BEFORE EXTRACTION WITH ALCOHOL	AFTER EXTRACTION WITH ALCOHOL
Nonsyphilitic pool	1/5	0	0
Syphilitic pool	1/5	1/64	0
Syphilitic pool	1/10	1/32	0
Syphilitic pool	1/20	1/8	0
Syphilitic pool	1/40	1/4	0
Syphilitic pool	1/80	0	0



In Table IV it is indicated that the reacting antigen was removed by the absolute alcohol extraction. Moreover, the alcoholic extract behaved like lipid antigen for syphilis and gave positive reactions with syphilitic sera.

When it was found that the nonspecific antigenic material was removed by alcohol extraction, it was necessary to determine the effect of alcohol on the antigenicity of a suspension of concentrated rickettsiae. A suspension of epidemic rickettsiae was therefore extracted with alcohol and titrated against 4 antibody units of a homologous convalescent serum. The alcohol-treated antigen was no longer active although the original material was antigenic at a dilution of 1/120.

*Specific and Nonspecific Activity of Crude Rickettsial Egg Antigens.*—Since Bengtson<sup>1, 2</sup> employed yolk sac antigens which had been purified by only one centrifugation and resuspension and since Reynolds and Pollard<sup>4</sup> recommended the use of commercial typhus vaccine as a complement-fixating antigen, it was considered of interest to test these antigens with syphilitic and non-syphilitic sera, using primary incubation of both one hour at 37.5° C. and eighteen hours at from 4 to 8° C. The results obtained are indicated in Table V.

TABLE V  
SPECIFIC AND NONSPECIFIC TITER OF CRUDE RICKETTSIAL EGG ANTIGENS

SERUM	SERUM DILUTION	COMMERCIAL TYPHUS VACCINE		CRUDE TYPE TYPHUS ANTIGEN	
		1 HR. AT 37.5° C.	18 HR. AT 4 TO 8° C.	1 HR. AT 37.5° C.	18 HR. AT 4 TO 8° C.
Epidemic typhus	(4 units)	1/16	1/64	1/32	>1/128
Nonsyphilitic pool	1/5	0	0	0	0
Syphilitic pool	1/5	0	1/128	0	1/128
Syphilitic pool	1/10	0	1/64	0	1/128
Syphilitic pool	1/20	0	<1/64	0	1/64
Syphilitic pool	1/40	0	1/32	0	1/32
Syphilitic pool	1/80	0	1/16	0	1/8

These results indicate that while the antigens tested did not give positive reactions with the pool of syphilitic sera employing the technique of a short period of incubation at 37.5° C. recommended by the authors, they nevertheless gave a considerable reaction with the same sera when tested by the more sensitive method of eighteen hours at from 4 to 8° C. Likewise, the degree of non-specific reaction of these antigens was well within the range of the specific titer with homologous antiserum and indicates that in serum titrations false positive fixation with the sera from syphilitic individuals would be obtained. In fact, when the sera from ten syphilitic individuals that were Kahn positive were tested with these antigens at eighteen hours at from 4 to 8° C., eight gave fixation of complement at a serum dilution ranging from 1/10 to 1/80, and two were negative at 1/10 dilution.

*The Absence of Nonspecific Reacting Substances in Rickettsial and Virus Antigens Prepared From Egg Material by Repeated Centrifugation and Resuspension.*—As was indicated previously, Plotz and I succeeded in eliminating nonspecific Wassermann type antigens by repeated centrifugation and washing of the spotted fever rickettsial suspensions. It was thought advisable at this time to recheck the activity of several of the purified antigens used at the Army Medical School against their homologous antisera as well as against the syph-

ilitic pool, using the various indicated periods of primary incubation. For this purpose purified antigens for epidemic typhus,<sup>5</sup> murine typhus,<sup>5</sup> and psittacosis<sup>6</sup> were titrated against 4 units of homologous antibody as well as against the positive and negative syphilitic pools used in a dilution of 1/5 and 1/10.

The results obtained are demonstrated in Table VI.

In these titrations, 2 full units of complement remained in each complement titration control with each period of primary incubation. It is evident from Table VI that a primary incubation of eighteen hours at from 4 to 8° C. is the more sensitive test; that is, complement is bound at a higher dilution of antigen when tested with a constant amount of antibody. Syphilitic sera, on the other hand, did not fix complement with these antigens at from 4 to 8° C.

TABLE VI  
SPECIFIC ANTIGEN TITERS AT VARYING PRIMARY INCUBATIONS

PURIFIED ANTIGENS	ANTISERUM	HIGHEST DILUTION OF ANTIGEN FIXING COMPLEMENT AT		
		1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.
Epidemic typhus	Epidemic (4 units)	1/50	1/60	1/120
	Syphilitic pool 1/5	0	0	0
Murine typhus	Murine (4 units)	1/50	1/60	1/160
	Syphilitic pool 1/5	0	0	0
Psittacosis	Psittacosis (4 units)	1/8	Not done *	1/24
	Syphilitic pool 1/5	0	Not done	0

#### DISCUSSION

This study emphasizes certain points which must be taken into consideration when employing complement-fixing antigens prepared from embryonic egg tissues. When relatively crude antigens are used, as suggested by Bengtson<sup>1,2</sup> and Reynolds and Pollard,<sup>4</sup> the technique of eighteen hours' incubation at from 4 to 8° C. cannot be used because nonspecific fixation of complement occurs with sera from syphilitic individuals. While the technique of one hour at 37.5° C. decreases this nonspecific reaction with syphilitic sera, the specific reaction is, at the same time, less sensitive in that lower antigen titers are obtained. With purified washed antigens, on the other hand, the sensitive technique of eighteen hours' incubation, at from 4 to 8° C., can be used without nonspecific reactions with syphilitic sera. It is for this reason that only purified rickettsial and psittacosis-lymphogranuloma venereum antigens are used in the Division of Virus and Rickettsial Diseases, Army Medical School.

#### SUMMARY

1. Normal yolk sac and normal embryo tissues contain an antigen which reacts with syphilitic sera. This reaction is more marked with a primary incubation of eighteen hours at from 4 to 8° C. than with a primary incubation of one hour at 37.5° C.

2. This nonspecific antigen is reduced approximately 50 per cent by ether extraction and is completely removed by extraction with absolute ethyl alcohol.

3. A simple centrifugation and resuspension of the tissue sediment reduces the nonspecific activity, while repeated washing and centrifugation completely eliminates it.

4. Crude rickettsial antigens that have undergone limited processing contain this nonspecific antigen in low titer. While these antigens did not give false positives with the syphilitic pool serum used at a primary incubation of one hour at 37.5° C., they did give strong false positives with the technique of eighteen hours' incubation at from 4 to 8° C. It is possible that similar false positive reactions may occur with other syphilitic sera and some antigens even when a primary incubation of one hour at 37.5° C. is used.

5. Rickettsial and elementary body antigens that have been purified by repeated washing and centrifugation no longer contain nonspecific complement-fixing antigen and may be used in tests employing the more sensitive technique of primary incubation of eighteen hours at from 4 to 8° C.

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# AN ALKALINE MEDIUM AND PROCEDURE FOR THE SELECTION OF DERMATOPHYTES IN THE PRESENCE OF SAPROPHYTIC FUNGI\*

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THE literature contains information showing the pathogenic fungi, that is, the dermatophytes, to be capable of growing in media of high initial pH values. Should the common saprophytic fungi be found not capable of growing in such media, it would then appear possible to apply this principle in a medium that would favor the dermatophytes over the rapidly growing saprophytic fungi.

This paper deals with the study of the growth of both dermatophytes and saprophytic fungi in the alkaline pH range and the development of a selective medium.

## HISTORICAL

The effects of pH on the growth of dermatophytes and saprophytes have been studied by many workers. In 1926 Keller<sup>1</sup> found that the "Kaufman-Wolf fungus" developed in media of a wide pH range from 6.8 to 12.0 with the optimum being from pH 6.8 to 7.0. He also found that *Epidermophyton inguinale* developed in a pH range of from 6 to 10 and grew best in a medium of pH 7.0.

Tate<sup>2</sup> refers to Verujsky (1887) as finding a neutral or slightly acid medium most favorable for growth with the optimum temperature being about 33° C., using *Tricophyton tonsurans* and *Achorion schoenleinii*. Tate,<sup>2</sup> using both Sabouraud's and synthetic media, found wide pH ranges for growth of the dermatophytes; the limit for growth on the acid side being from 3.0 to 4.0, while it was "beyond pH 8.0" on the alkaline side, the optimum pH being about 6.0 to 7.0. He stated that all the dermatophytes were found to have an active proteolytic enzyme which acts in an alkaline medium and can hydrolyze intact proteins (casein) with the production of free amino acids. This enzyme resembles trypsin, no pepsin being found. In *Aspergillus niger* the proteolytic enzyme acts in a strongly acid medium and resembles pepsin.

In 1929 Kadisch<sup>3</sup> found the best growth of the dermatophytes on Sabouraud's maltose agar, on 3 per cent peptone agar, and on Grütz-glycerine agar occurred at from pH 7.2 to 7.6. He found a tendency for cultures started on the acid side to become alkaline as a result of fungal activity. He also<sup>4</sup> found 37° C. unfavorable for *Achorion gypseum*. Biltris<sup>5</sup> grew *Trichophyton gypseum* asteroides on peptone 3 per cent and agar 18 per cent, with the medium at initial pH values of 3.8, 6.0, and 11. No fuscaux occurred at pH 11.

Mallinekrodt-Haupt<sup>6</sup> found that *T. gypseum* grown in buffered solutions for several months changed an acid medium to basic and a basic solution to almost neutral. This was true also for *Achorion quinckeanum*, but it made the basic

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medium more acid (pH 6.10 to 6.92 from pH 8.0). When these fungi were started on media at pH 6.01 to 7.19 they changed the pH to the basic side of neutrality, while *Penicillium glaucum* and "Rosa Lufthebe" made the same media more acid. These differences are explained by the ability of the pathogens to break down the protein source of nitrogen.

In 1930 Talice<sup>7</sup> presented data which showed *Sabouraudites granulosis* (on Sabouraud's proof medium) able to grow at a minimum of pH 4 with its maximum above 9.6. Both *Penicillium citrinum* and *Rhizopus nigricans* were able to grow at pH 2.2, with their minimum pH being below this. Their maximum pH was, as with *S. granulosis*, above 9.6. The optimum of *S. granulosis* was given as pH 6 to 7.

Levin and Silvers<sup>8</sup> found the reaction of the fourth interdigital space of the foot to vary in different individuals from pH 6.30 to 7.85. The higher value was obtained from a case of marked dermatophytosis. Another high value of pH 7.65 was obtained, and this, too, was from an active case of dermatophytosis of the foot.

Mallinekrodt-Haupt,<sup>9</sup> in discussing the change in reaction which the molds produce in the growth medium, stated that true dermatophytes, with the exception of the *Epidermophyton* fungi, showed strong tendencies toward the formation of alkali, while most of the saprophytic *Hyphomyces* produced acid.

Cerutti<sup>10</sup> stated that *T. gypsum* always gives an alkaline reaction during its development, while the "Achorion of Schoenlein" and the "Sporotrichum of Gougerot and Sehenek" give an acid reaction at the beginning that later turns toward alkalinity.

Belisario<sup>11</sup> stated that "the predilection of mycological infections for the palms and soles is due to the alkaline tendency of the sweat in these areas." Where sebaceous glands are richly supplied, mycotic infections are rarely found, he states, and "... the hyperacidity of the sebaceous glands at puberty, through increasing the hydrogen-ion concentration of the scalp sweat, may afford a feasible explanation of the disappearance of certain small-spore ringworms at that age."

Williams<sup>12</sup> noted an apparent slight increase in growth of *T. gypsum* when the pH of the medium (cysteine) was raised from 5.4 to 6.6.

Peck and Rosenfeld,<sup>13</sup> using "Sabouraud's bouillon" unbuffered, obtained growth of *T. gypsum* in the range of pH 3.4 to pH 10.0 inclusive. No growth occurred at pH 3.0. Using McIlvaine's buffers, they obtained growth from pH 3.4 to pH 9.0. Plates poured with buffered media showed growth throughout the whole pH range (from 4 to 10) studied, but *T. gypsum* showed definite retardation at all pH values except pH 7.0 and 8.0. *E. inguinale* was retarded through the series. *T. gypsum* was found to increase the pH once growth started, and at the end of three weeks without buffer raised the pH from 5.0 to pH 7.6, whereas with buffer the pH rose to 6.5. As all of the nonbuffered solutions rose to a pH of from 7.6 to 8.0, Peck and Rosenfeld concluded that the "optimum" pH range for *T. gypsum* must lie here. They believe that the presence of a buffer, when it interferes with the production of a pH value optimum for growth, retards the growth of the organism and explains the less vigorous growth obtained with buffered solutions as compared with that obtained in nonbuffered solutions.

In media with no buffer and pH values from 4.0-8.0, Peck and Rosenfeld obtained a change to a more alkaline pH using *T. gypsum*; at pH 8.0 there was

only a slight increase in the pH value, and at pH 9.0 and 10.0 a change to a more acid reaction took place, the pH dropping to 8.08 and 7.87, respectively. With buffers the pH increased less and dropped more (except at pH 3.4), there being a sharp drop from pH 8.0, 9.0, and 10.0.

#### METHODS

*Cultures.*—The saprophytic fungi used in this study consisted of *A. niger* No. 7, *A. niger* No. 12 (isolated from worn shoes), *R. nigricans*, *Penicillium* species No. 8, No. 9, No. 10, and No. 11, with the latter three species being isolated from worn shoes. All three genera are the ones commonly occurring on shoes, floors, etc. The dermatophytes used were, *Epidermophyton floccosum*, *Tricophyton interdigitale*, *Tricophyton purpureum* and *T. gypsum*.

In the early part of the research a piece of colony growth was streaked over the surface of the agar or it was shaken with water and then poured with the test media. Later, suspensions of the cultures were made with water, and both streaked and poured plates were made, using the suspension as the inoculum.

*Media and Incubation.*—Sabouraud's maltose agar of the following composition was used throughout this study: Bacto-peptone, 10 Gm.; bacto-maltose, 40 Gm.; bacto-agar, 15 Gm.; and water, 1,000 c.c. No adjustment of the pH was made prior to sterilization at 15 pounds steam pressure for twenty minutes.

Immediately before plates were poured with this medium the pH was adjusted by the addition of approximately 1.0 N sterile NaOH to the bottles of melted agar. Adjustment to pH 10.5 was made by the use of LaMotte purple. This was later checked with a Beckman pH meter. The media were not buffered, and it is recognized that the pH was lowered merely upon standing. Thus the pH values given are the initial values only, and the term alkaline medium refers to Sabouraud's dextrose or maltose agar adjusted to an initial pH of 10.5

#### EXPERIMENTAL

*Effects of Increased Alkalinity.*—As an acid pH is used to inhibit bacteria while permitting mold growth, the effects of using a low pH of 3.0 and a high pH of 8.0 on the growth of pathogenic and nonpathogenic fungi were studied. Plates of Sabouraud's maltose agar were adjusted to a pH of 3.0 with lactic acid and to pH 8.0 with sodium hydroxide and streaked with saprophytes and dermatophytes. After incubation at 34° C. for fifteen days, both *A. niger* No. 7 and *Penicillium* sp. No. 8 had failed to show growth at either pH. However, with three dermatophytes (*T. interdigitale*, *T. gypsum* and *T. purpureum*) growth occurred in three and one-half days on the pH 8.0 medium but did not occur on the pH 3.0 medium. Therefore, pH values of 8.5 and 9.0 were tested along with pH 8.0.

Since the combined effects of temperature and pH are likely to be more pronounced than either one alone, plates of Sabouraud's maltose agar adjusted to the desired pH values were streaked with various fungi and incubated at 34° C. and at 37° C. In Table I, Part A, it is indicated that the temperatures used do not influence growth (with the exception of *Penicillium* sp. No. 8) until the pH of the medium is raised to 9.0. At this pH, all of the molds tested, with the exception of *A. niger* No. 7, gave less growth at 37° C. than at 34° C. At 37° C.

and pH 9.0, *R. nigricans* was completely inhibited, while *Penicillium* sp. No. 8 took longer to initiate growth at 37° C. than it did at 34° C. regardless of the pH used. As the pH increased from 8.0 to 8.5 to 9.0 and with an incubation temperature of 34° C., it is seen that none of the dermatophytes was inhibited.

TABLE I

GROWTH OF DERMATOPHYTES AND SAPROPHYTES *Streaked* ON SABOURAUD'S MALTOSE AGAR AT VARIOUS HYDROGEN-ION CONCENTRATIONS

INCUBATION AT 34° AND 37° C.

Part A—Media Adjusted to Initial pH of 8.0, 8.5, and 9.0

CULTURES	INCUBATION TEMPERATURE ° C.	SABOURAUD'S MALTOSE AGAR OF					
		pH 8.0		pH 8.5		pH 9.0	
		DAYS INCUBATION*		DAYS INCUBATION		DAYS INCUBATION	
		4	6	4	6	4	6
<i>T. interdigitale</i>	34	+++	+++	+++	+++	+++	+++
	37	+++	+++	+++	+++	++	++
<i>T. gypsum</i>	34	+++	+++	+++	+++	+++	+++
	37	+++	+++	+++	+++	++	++
<i>T. purpureum</i>	34	+++	+++	+++	+++	+++	+++
	37	+++	+++	+++	+++	++	++
<i>R. nigricans</i>	34	+++	+++	+++	+++	+	+
	37	+++	+++	+++	+++	-	-
<i>A. niger</i> No. 7	34	+++	+++	+++	+++	++	++
	37	+++	+++	+++	+++	++	++
<i>Penicillium</i> sp. No. 8	34	+	+	+	+	++	++
	37	-	+	-	+	-	±*

-, No growth.

+, Slight growth.

++, Good growth.

+++ , Very good growth.

++++, Excellent growth.

±, Growth doubtful as to presence or type (dermatophyte or saprophyte).

Blank spaces indicate plates were not read.

p, Pinpoint "colonies."

\*All plates examined also after eight days and showed no change, except *Penicillium* sp. No. 8 was definitely + at 37° C. pH 9.0.

TABLE II

GROWTH OF DERMATOPHYTES AND SAPROPHYTES *Streaked* ON SABOURAUD'S MALTOSE AGAR AT VARIOUS HYDROGEN-ION CONCENTRATIONS

INCUBATION AT 34° AND 37° C.

Part B—Media Adjusted to Initial pH of 9.0, 9.5, and 10.5

CULTURES	INCUBATION TEMPERATURE ° C.	SABOURAUD'S MALTOSE AGAR OF											
		pH 9.0				pH 9.5				pH 10.5			
		DAYS INCUBATION				DAYS INCUBATION				DAYS INCUBATION			
		2	3	7	10	2	3	7	10	2	3	7	10
<i>T. interdigitale</i>	34	+++	++++	++++	++++	+++	++++	++++	++++	+++	+++		
	37	+++	++++	++++	++++	++	+++	++++	++++	±p	+	+++	+++
<i>T. gypsum</i>	34	+++	++++	++++	++++	+++	++++	++++	++++	+++	+++		
	37	+++	++++	++++	++++	++	+++	++++	++++	++	+++	+++	+++
<i>T. purpureum</i>	34	+	++	+++	+++	+	+++			+	+++	+++	
	37	+	+++	+++		+	+++	+++		+	++	+++	+++
<i>R. nigricans</i>	34	+	+++			-	-	+		-	-	-	-
	37	++	+++	++		-	-	+	++	-	-	+	
<i>A. niger</i> No. 7	34	+++	+++			+	+	++		-	+	++	
	37	++	++	++		++	++	++		-	±	+++	
<i>Penicillium</i> sp. No. 8	34	++	++	+++	++++	++	++	++	++++	+	+	++	+++
	37	-	-	+		±p	+	++		-	-	++	

TABLE I

GROWTH OF DERMATOPHYTES AND SAPROPHYTES *Streaked* ON SABOURAUD'S MALTOSÉ AGAR AT VARIOUS HYDROGEN-ION CONCENTRATIONS

INCUBATION AT 34° AND 37° C.

Part C—Media Adjusted to Initial pH of 10.5 and 11.5

CULTURES	INCUBATION TEMPERATURE ° C.	SABOURAUD'S MALTOSÉ AGAR OF							
		pH 10.5				pH 11.5			
		DAYS INCUBATION				DAYS INCUBATION			
		3	4	7	11	3	4	7	11
T. interdigitale	34	++++	++++	++++	++++	++++	++++		
	37	-	+p	+	++	-	-	+	+
T. gypsum	34	++++	++++	++++	++++	++++	++++		
	37	-	+p	+	++	-	+	+	+
T. purpureum	34	+++	+++			+++	+++		
	37	-	+p	+	+++	-	-	+	+
R. nigricans	34	-	-	+++		-	-	-	
	37	-	-	-	-	-	-	-	++
A. niger No. 7	34	+++	+++	-		++	++		
	37	-	-	+		-	-	+	+
Penicillium sp. No. 8	34	++	++			++	++		
	37	++	++	+++		+	+	+++	+++

The saprophytic fungi, on the other hand, showed less growth at the high pH of 9.0. *Penicillium* sp. No. 8 did not show less growth at the high pH but showed a slight increase in growth in this particular experiment. These results indicated possible further advantages in a still more alkaline medium. The pH was increased to 9.0, 9.5, and 10.5 with plates being streaked and incubated at 34° C. and 37° C. The results obtained (Table I, Part B) show the pathogenic molds growing about as well at pH 10.5 as at pH 9.0 with 34° C. as the incubation temperature. At the same temperature and at pH 10.5, *R. nigricans* and *A. niger* No. 7 were markedly inhibited (Fig. 1). A prolonged incubation period of fourteen days showed good growth with *R. nigricans* on the pH 10.5 medium at 34° C. but showed no growth in seventeen days on the same medium at 37° C.

The inhibition of the saprophytic fungi at pH 10.5 is greater at 37° C., but the greater inhibition of the dermatophytes at this temperature than at 34° C. outweighs the beneficial effects of the higher temperature. The pH 10.5 medium showed a distinct selective action against the saprophytic fungi by inhibiting them to a much greater extent than the dermatophytes, so this experiment using Sabouraud's maltose agar adjusted to initial pH values of 9.0, 9.5, and 10.5 with incubation at 34° C. and 37° C. was repeated with the result that pH 10.5 at 34° C. again showed the best selective action.

Inasmuch as a pH of 10.5 proved effective toward attaining the desired goal of a medium capable of inhibiting the saprophytic fungi while allowing the dermatophytes to grow, it was thought that a higher pH might prove to be more effective. Plates of Sabouraud's maltose agar adjusted to pH 10.5 and 11.5 were streaked individually with saprophytic fungi and dermatophytes with incubation at 34° and 37° C. (Table I, Part C). There was no significant difference between growth at pH 10.5 and pH 11.5 at either 34° C. or 37° C. However, it can be seen that the best selection of dermatophytes over saprophytic fungi was at 34° C.



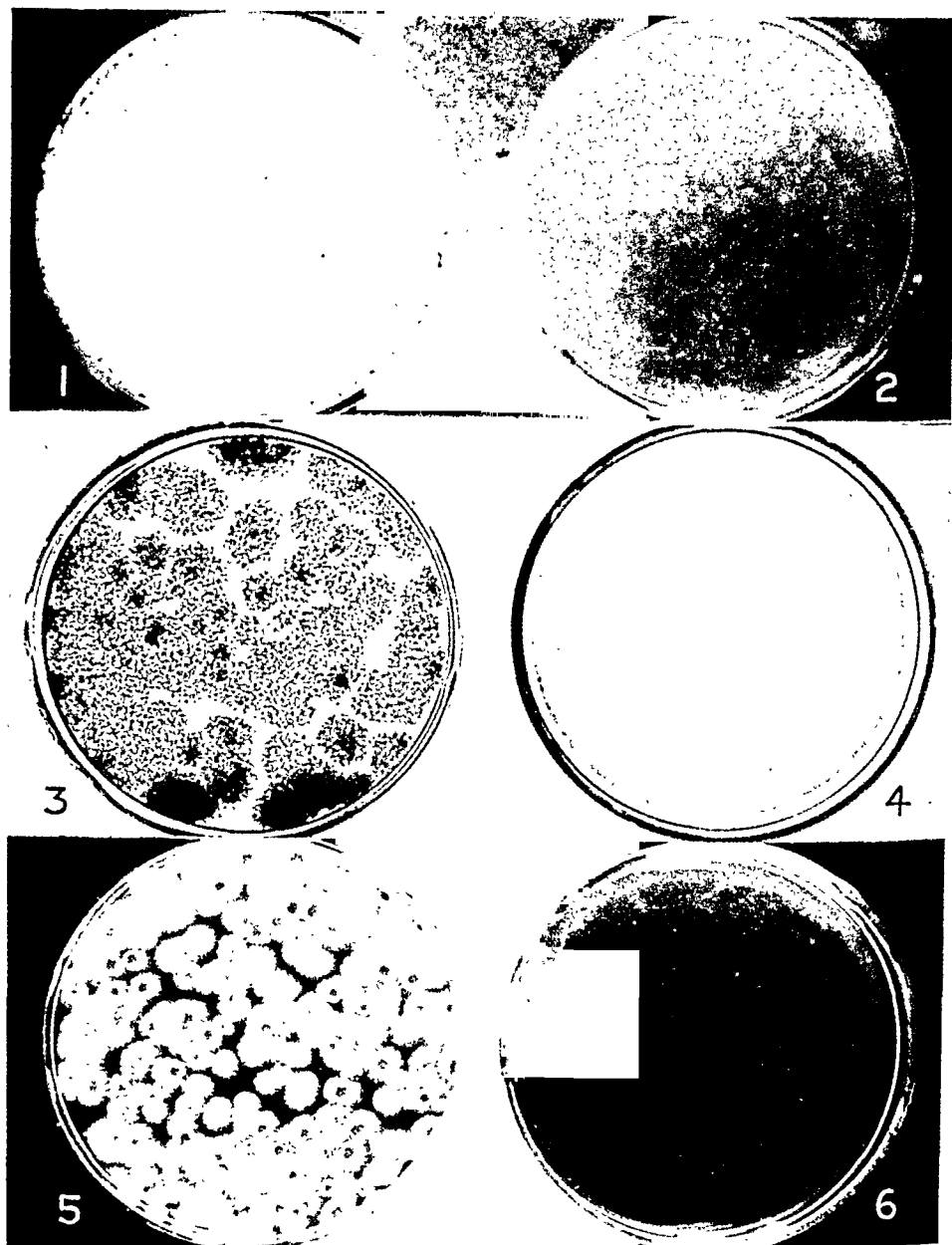


Fig. 1.—A comparison of Sabouraud's dextrose agar and the alkaline medium inoculated with pure cultures. Incubation, three days at 34°C.

Plates 1, 3, and 5 were poured with Sabouraud's dextrose agar, while 2, 4, and 6 were poured with the alkaline medium.

Plates 1 and 2 contain the same inoculum of *R. nigricans*.

Plates 3 and 4 contain the same inoculum of *A. niger* No. 7.

Plates 5 and 6 contain the same inoculum of *Penicillium* sp. No. 34.

*Streaked vs. Poured Plates.*—The test plates in all experiments so far were streaked. However, as one purpose of this study was to devise a method for the isolation of dermatophytes from shoes, a procedure based on streaking was considered inferior to that of inoculating plates with a suspension from the leather and pouring with agar. Therefore, results obtained by use of poured and streaked plates were compared.

Pure cultures of four strains of dermatophytes and seven strains of saprophytic fungi were streaked and poured individually with the alkaline medium and were incubated at 37° C. The results obtained (Table II) indicate that there was slightly better growth in the poured plates. Therefore, a comparison was made between the two techniques, using pathogenic fungi individually mixed with *Penicillium* sp. No. 8. The alkaline medium was streaked and poured with the mixed inocula (the same inoculum was used for both).

TABLE II

A COMPARISON OF GROWTH OF PURE CULTURES ON *Streaked* AND *Poured* PLATES USING THE ALKALINE MEDIUM (MALTOSE)

INCUBATION AT 37° C.

CULTURES	POURED							STREAKED						
	DAYS INCUBATION							DAYS INCUBATION						
	1	2	3	4	5	7	9*	1	2	3	4	5	7	9*
<i>E. floccosum</i>	+	+	++	+++	+++			-	-	+	+	+++	+++	
<i>T. interdigitale</i>	-	+	+++	++++	++++	++++	++++	-	+	+++	+++			
<i>T. gypsum</i>	-	+	+++	++++	++++	++++	++++	-	++	+++	+++	++++	++++	++++
<i>T. purpureum</i>	+	+	++	++++	++++	++++	++++	-	+	++	+++			
<i>R. nigricans</i>	-	-	-	-	-	+	++	-	-	-	-	-	+	++
<i>A. niger</i> No. 7	-	-	++	+++	++++	++++	++++	-	+	++	+++			
<i>Penicillium</i> sp. No. 8	+	+	++	+++				+	+	+	++	++		
<i>Penicillium</i> sp. No. 9	-	+	+	+	++	++	++	-	-	-	-	-	-	+++ 15 days
<i>Penicillium</i> sp. No. 10	-	-	-	-	+	++	++	-	-	-	-	-	++	+++
<i>Penicillium</i> sp. No. 11	-	-	-	-	++	++		-	-	-	+	+	++	
<i>A. niger</i> No. 12	++	+++	++++	++++	++++	++++	++++	-	++++	++++	++++	++++	++++	++++

\*Plates examined after fifteen days showed no change other than as noted

TABLE III

A COMPARISON BETWEEN *Streaked* AND *Poured* PLATES OF ALKALINE AGAR (MALTOSE) WITH MIXED CULTURES OF DERMATOPHYTES PLUS *PENICILLIUM* SPECIES NO. 8

INCUBATION AT 37° C.

CULTURES	TYPE OF GROWTH	STREAKED				POURED			
		DAYS INCUBATION				DAYS INCUBATION			
		1	2	3	4	1	2	3	4
<i>T. interdigitale</i>	P	-	±	±	+	-	±	+	+
	NP	-	±	±	+++	-	±	-	±
<i>T. gypsum</i>	P	-	±	±	+	-	±	+	±
	NP	-	±	±	+++	-	±	-	±
<i>T. purpureum</i>	P	-	±	±	+	-	±	-	+
	NP	-	±	±	+++	-	±	+	±

P, Pathogen (dermatophytes).

NP, Nonpathogen (saprophytes).

The results are given in Table III. All pathogenic fungi showed a slight growth on the streaked alkaline plates after four days' incubation, while *Penicillium* showed a heavy growth on the same plates. On the poured plates, growth was doubtful or poor for both the dermatophytes and *Penicillium*. The poured plates did not produce materially less growth of the dermatophytes compared with that obtained by use of the streaked method but did show only very slight *Penicillium* growth when the streaked method showed a very good growth. The advantage of the poured over the streaked plate method in inhibiting the saprophytic fungi while affecting the dermatophytes either not at all or to a less degree is definite. Using this method it is possible to inhibit *Penicillium* to

TABLE IV

GROWTH OF DERMATOPHYTES AND SAPROPHYTES IN PLATES *Poured* With SABOURAUD'S MALTOSÉ AGAR AT VARIOUS HYDROGEN-ION CONCENTRATIONS

INCUBATION AT 31° C.

## Part A

CULTURES	SABOURAUD'S MALTOSÉ AGAR OF									
	pH 5.5					pH 9.5				
	DAYS INCUBATION					DAYS INCUBATION				
	3	4	5	6	7*	3	4	5	6	7*
<i>E. floccosum</i>	-	+	++	++	+++	+	++	+++		
<i>T. interdigitale</i>	++	++++	++++	++++	++++	+	+++	++++	+++	++++
<i>T. gypsum</i>	++	++++	++++	++++	++++	+++	++++	++++	+++	++++
<i>T. purpureum</i>	++	+++	++++	++++	++++	++	+++	++++	+++	++++
<i>R. nigricans</i>	++++	++++	++++	++++	++++	-	-	++		
<i>A. niger</i> No. 7	+++	+++				+++	+++	+++		
<i>Penicillium</i> sp. No. 8	+++	+++				++	++	++		
<i>Penicillium</i> sp. No. 9	+	++++	++++	++++	++++	-	+	++	++	++
<i>Penicillium</i> sp. No. 10	+	++++	++++	++++	++++	-	-	+	+	+
<i>Penicillium</i> sp. No. 11	++++	++++	++++	++++	++++	-	-	+	+	+
<i>A. niger</i> No. 12	+++	+++	+++	+++	+++	+++	+++	+++		

\*Plates examined after twelve days showed no change, except *Penicillium* sp. No. 9, No. 10, and No. 11 were +++, ++, and +++, respectively, at pH 9.5.

TABLE IV

GROWTH OF DERMATOPHYTES AND SAPROPHYTES IN PLATES *Poured* With SABOURAUD'S MALTOSÉ AGAR AT VARIOUS HYDROGEN-ION CONCENTRATIONS

INCUBATION AT 34° C.

## Part B

CULTURES	SABOURAUD'S MALTOSÉ AGAR OF									
	pH 10.0					pH 10.5				
	DAYS INCUBATION					DAYS INCUBATION				
	3	4	5	6	7*	3	4	5	6	7*
<i>E. floccosum</i>	+	+	++	+++		+	+	++	++	++
<i>T. interdigitale</i>	+	++	++++	++++	++++	+	+	+	++	+++
<i>T. gypsum</i>	++	++++	++++	++++	++++	+	+++	++++	++++	++++
<i>T. purpureum</i>	+	++	++			+	+	+++	+++	+++
<i>R. nigricans</i>	-	-	-	-	++	-	-	-	-	-
<i>A. niger</i> No. 7	+	+++	+++			-	+	+	++	++
<i>Penicillium</i> sp. No. 8	+++	+++	+++			++	++	+++	+++	+++
<i>Penicillium</i> sp. No. 9	+	+	+	++		-	-	-	-	-
<i>Penicillium</i> sp. No. 10	-	+	+	+	++	-	-	-	-	-
<i>Penicillium</i> sp. No. 11	-	-	-	+	+	-	-	-	-	-
<i>A. niger</i> No. 12	++	++++	++++	++++	++++	+	++	++++	++++	++++

\*Plates examined after twelve days showed no change except as follows: at pH 10.0, *R. nigricans* was +++, *Penicillium* sp. No. 10 was ++, and *Penicillium* sp. No. 11 was +++; at pH 10.5, *R. nigricans* and *Penicillium* sp. No. 9 were ++, *Penicillium* No. 10 was +, and *Penicillium* sp. No. 11 was +++.

an appreciable extent while the dermatophytes are not so affected. Although the previous pure culture experiment (Table II) showed little difference in growth between streaked and poured plates, the latter experiment (Table III) does show the poured plate to be superior in selecting pathogenic growth from a mixture of dermatophytes with *Penicillium* sp. No. 8.

Using 34° C. as the temperature of incubation, a series of Sabouraud's maltose agar poured plates was prepared with the pH at 5.5 (unadjusted) and adjusted to pH 9.5, 10.0, and 10.5. The results obtained (Table IV, Parts A and B) show the efficiency of the alkaline medium (pH 10.5) in restraining growth of saprophytic fungi. In comparing the pH 5.5 with the pH 10.5 results, it is seen that the dermatophytes were inhibited by the high pH although less on extended incubation (see also Figs. 2 and 3). Nevertheless, the effect of the pH 10.5 medium upon the saprophytic fungi is more pronounced. *R. nigricans* and *Penicillium* sp. No. 9, No. 10, and No. 11 were completely inhibited for seven days, thus giving the pathogenic fungi ample time to produce visible growth. Six days are sufficient for pathogenic growth to appear on the alkaline medium at 34° C. On the unadjusted medium the saprophytic fungi showed a fairly rapid growth which would have overgrown any dermatophytes present. *A. niger* No. 7 was inhibited for three days at pH 10.5, showed poor growth on the fourth day and fair growth from then until the seventh day, while at the unadjusted pH of 5.5 good growth was obtained on two days' incubation. Here again, a dermatophyte would have a chance to grow in the alkaline medium but not on the unadjusted medium. *Penicillium* sp. No. 8 was inhibited in the alkaline medium, and although many colonies appeared, the high pH kept them from spreading over the plate. Instead, the *Penicillium* colonies remained small and restricted, although an occasional colony would show spreading. Therefore, any dermatophytes in the presence of this saprophytic fungus on an alkaline plate likewise would have a chance to appear, while on Sabouraud's maltose agar of unadjusted pH the rapid, spreading growth of *Penicillium* sp. No. 8 would greatly reduce any chance of growth by the dermatophytes. The remaining saprophytic fungus, *A. niger* No. 12, was inhibited by the 10.5 medium, for very good growth was obtained after two days' incubation at 34° C. on pH 5.5, while it took five days of incubation to produce the same results with the alkaline medium. On this alkaline medium, *A. niger* No. 12 showed no growth after two days' incubation and only fair growth after four days' incubation at 34° C. The results at pH 9.5 and 10.0 are similar to, but not as striking as, those obtained at pH 10.5. Therefore, it is seen that the use of the alkaline medium incubated at 34° C. will inhibit saprophytic fungi much more strongly than the pathogenic fungi.

It was noted, in the platings from numbers of shoes, that the predominant saprophytic fungi were *Aspergillus* and green *Penicillium*. Cultures of these two genera were combined individually with *T. gypsum* and plates poured with both unadjusted Sabouraud's maltose agar and the alkaline medium, the purpose being to test the selective action of the alkaline medium with mixed cultures and to corroborate the results and conclusions of the previous experiment. The results are given in Table V. Using *T. gypsum* mixed with *A. niger* No. 7, the *Aspergillus* grew rapidly and completely covered the plate of unadjusted



Fig. 2.

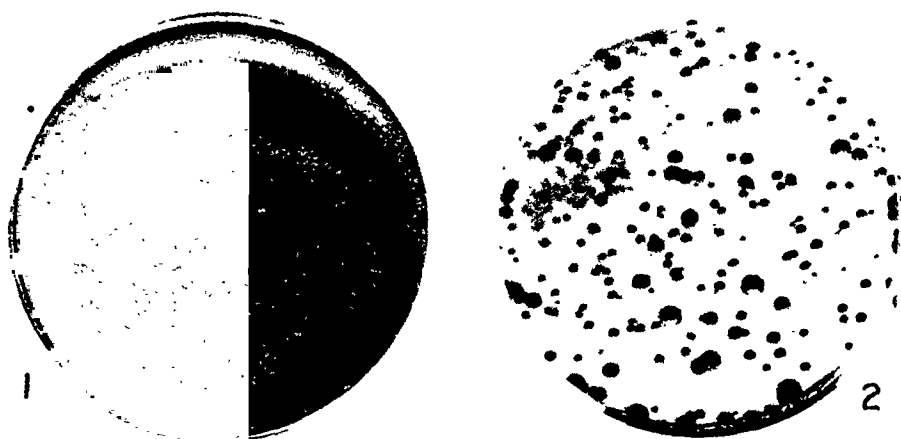


Fig. 3.

Figs. 2 and 3.—A comparison of Sabouraud's dextrose agar and the alkaline medium inoculated with a pure culture of *T. mentagrophytes* and incubated for three and for five and one-half days at 34°C., respectively.

Plates 1 were poured with Sabouraud's dextrose agar, while plates 2 were poured with the alkaline medium.

Fig. 2.—Inoculum of approximately 40. Incubation, three days.

Fig. 3.—Inoculum of approximately 40,000. Incubation, five and one-half days.

Sabouraud's agar, and after five days' incubation no growth of the dermatophyte was visible on the medium. The same inoculum plated with the alkaline medium showed many colonies of *T. gypsum* and only a few of *Aspergillus*. When *Penicillium* sp. No. 8 was used as the saprophyte, no growth of *T. gypsum* appeared in either the alkaline or the unadjusted medium because of heavy growth by the *Penicillium* which covered the entire surface of the agar. It is interesting to note (Tables II and IV) that the different strains of *Penicillium* used

TABLE V

THE SELECTIVE ACTION OF THE ALKALINE MEDIUM UPON MIXTURES OF *T. GYPSEUM* PLUS *A. NIGER* NO. 7 AND *T. GYPSEUM* PLUS *PENICILLIUM* SP. NO. 8 (PLATES POURED)

INCUBATION, FIVE DAYS AT 34° C.

CULTURES	SABOURAUD'S MALTOSE AGAR (pH 5.5)	ALKALINE MEDIUM
<i>T. gypseum</i> alone	Colonies small, white, and spreading	Colonies smaller, white, and fewer in number
<i>A. niger</i> No. 7 alone	Spreading black growth	Colonies are separate, yellow with few black spores
<i>Penicillium</i> sp. No. 8 alone	Numerous green colonies	Colonies green, smaller, and less in number
<i>T. gypseum</i> + <i>A. niger</i> No. 7	Plate black with <i>A. niger</i> ; no pathogens visible	Few <i>Aspergilli</i> ; pathogen colonies present
<i>T. gypseum</i> + <i>Penicillium</i> sp. No. 8	All green; no pathogens	All green; no pathogens

varied in their reaction on the alkaline medium. Strain No. 8, used in the experiment just described (Table V), was the one which had previously shown the best growth in this medium. Tate (see under Historical) found that the dermatophytes have a trypsinlike enzyme, while a saprophytic fungus as *A. niger* does not. As trypsin is effective in alkaline substrates, it may explain why the dermatophytes are able to grow comparatively well on pH 10.5 media, while the saprophytes are not. Also, the nature and extent of the enzyme systems may account for the variable reactions of the *Penicillium* strains used. On this basis, *Penicillium* sp. No. 8 may very well have a strong trypsinlike enzyme system and may, therefore, approach a pathogenic strain

#### DISCUSSION

The study of the growth of dermatophytes and saprophytic fungi on Sabouraud's maltose agar of increasing alkaline pH concentrations has shown that the dermatophytes are capable of growing on media initially adjusted up to pH 11.5, whereas the saprophytes were largely inhibited at values above pH 9.0. The pH at which the best inhibition of saprophytes with the least comparative inhibition of dermatophytes occurred was at pH 10.5. It was found that poured plates were superior to streaked plates in inhibiting the saprophytic fungi and, therefore, in giving the best selection of dermatophytes.

A temperature of 37° C., while more effective than 34° C. in eliminating or reducing saprophytic growth, was found to be more inhibitory to the dermatophytes. Also, 34° C. is to be preferred over a fluctuating room temperature.

The experimental work also showed that an incubation period of five and one-half days at 34° C., using poured plates of the alkaline medium (Sabouraud's maltose agar of pH 10.5), gave the best results. If plates containing colonies of saprophytic fungi are incubated longer than five and one-half days, these fungi may overgrow the dermatophytes. However, plates which after five and one-half days are negative for pathogenic fungi, or on which there is no overgrowth of possible dermatophytic colonies, should be incubated for about ten days, with the plates being observed daily. Colonies of mold growth that are thought to be pathogenic should be transferred to ordinary Sabouraud's dextrose agar (or other suitable media) for purification and examination.

## THERAPEUTIC EFFECTIVENESS OF PENICILLIN IN TREATMENT OF VINCENT'S STOMATITIS AND ITS FAILURE TO INFLUENCE FAVORABLY CERTAIN OTHER MEDICAL CONDITIONS

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MEDICAL CORPS, ARMY OF THE UNITED STATES

THE Surgeon General, U. S. Army, has encouraged some of the general hospitals to use penicillin in various medical diseases. Such an exploitation of this relatively new therapeutic agent has been undertaken obviously for the purpose of establishing accurate limits demarcating its value. In this hospital, an attempt has been made, therefore, to treat a variety of diseases with the drug. While the experience in general confirms that of other clinical investigators, it would seem of value to record the good therapeutic results obtained in the treatment of Vincent's stomatitis and the failure of penicillin to influence favorably a number of other medical diseases.

Forty-three patients with Vincent's stomatitis were treated with from 600,000 to 1,000,000 units of penicillin. The antibiotic was given intramuscularly in doses of 25,000 units each three hours. The average total dose was 721,000 units. All of the patients were cured. After forty-eight hours of therapy, it was usually impossible to find fusiform bacilli and spirochetes except in those in whom marked dental caries existed. In these patients, furthermore, the organisms disappeared rapidly after dental prophylaxis. It is interesting that oral temperatures frequently rose from a normal level to from 99.6 to above 101° F. during the time that penicillin was being administered. This phenomenon is unexplained and was not noted during the treatment of other diseases.

Three patients with chronic and recurring furunculosis were treated with doses of from 630,000 to 1,475,000 units. There was evidence of prompt improvement. Relapse, however, has since occurred in one patient. Penicillin, in our opinion, should be regarded only as a palliative or temporarily helpful agent in the treatment of recurring boils. It was injected for an average of eleven days.

Eleven patients with *acne vulgaris* were treated with doses of penicillin ranging from 2,750,000 to 3,650,000 units. While an occasional patient showed signs suggestive of improvement, none was cured nor markedly improved. Ten patients with staphylococcal dermatitis were treated with from 2 to 3 million units. Again some showed evidence of improvement but relapse was frequent; it is felt that penicillin has very little, if any, curative value in this skin disease. Penicillin was injected for an average of eighteen days.

In seven cases of asthma which were thoroughly studied and were thought to be bacterial in origin the patients were treated intensively with doses of

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from 1 to 5 million units. Various strains of streptococci and staphylococci were recovered from the paranasal sinuses of these patients. Initially there seemed to be some evidence of improvement, but it was not sustained. Penicillin was injected for an average of fourteen days.

Five patients with malaria were treated with doses varying from 2 to 5 million units of penicillin. While there was some shifting of the white blood count and a disturbance in the periodicity of the paroxysms, the therapeutic agent had no curative value. It should be added that these five patients have been treated subsequently with antimalarial agents in relatively small doses and have responded promptly. They have had no recurrences after an observation period of six months. It is conceivable that penicillin might possibly have caused them to be more responsive to antimalarial drugs.

Five patients with rheumatic fever were treated with entirely negative results. The total dose used varied from 1,400,000 to 2,600,000 units. Two patients with subacute bacterial endocarditis, proved at autopsy, received 5,600,000 units and 5,009,000 units during a period of twenty-eight and twenty-five days, respectively. Although the blood cultures became negative while penicillin was being administered, they became positive again when treatment was stopped.

One patient with disseminated lupus received 6 million units over a long period of time. On two occasions it was given for two weeks because of pneumonic complications and for an additional two weeks for the basic condition, making a total of 6 million units during a period of six weeks. The patient manifested recurrent bouts of painful swollen joints, bilateral pleurisy, pericarditis, two attacks of streptococcic pneumonia (alpha and beta hemolytic), three attacks of jaundice, transient skin manifestations characteristic of lupus erythematosus, several bouts of unexplained diarrhea, a more or less continuous low-grade fever, weight loss, and anemia. At no time was there any evidence of clinical improvement with penicillin.

One or more patients with one of the following diseases failed to respond to the administration of penicillin: chronic pyelonephritis (given over 2 million units; aerobic and anaerobic nonhemolytic streptococcus and nonhemolytic staphylococcus isolated from urine), diphtheria; mumps; eczema; folliculitis; dermatitis herpetiformis; acute myeloid leukemia; and follicular lymphoblastoma (Brill-Symmer's disease). One patient with a peritonsillar abscess failed to improve when given penicillin until the abscess was drained surgically. Several of the patients treated with penicillin also had trichophytosis; this latter lesion showed no improvement.

As for reactions, there was noted in only three patients a mild to moderate urticaria which disappeared within a few days during penicillin therapy.

#### SUMMARY

Forty-three patients with Vincent's stomatitis showed prompt recovery when treated with penicillin. The failure of this chemotherapeutic agent to influence favorably a number of other medical diseases is recorded.



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# EVALUATION OF TECHNIQUES USED IN THE DIAGNOSIS OF ENTEROZOIC PARASITISM IN CHILDREN

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IN THIS paper is described the comparative value of several techniques used in a survey designed to determine the incidence of enterozoic parasitism in children.<sup>1</sup> In the 512 children between the ages of 6 weeks and 12 years included in the survey, a total of 646 specific parasites was found. The average incidence, therefore, was approximately 1.3 parasites per child. The frequency with which the different parasites were found with the combined methods was as follows: *Entamoeba histolytica*, 8.0 per cent; *Entamoeba coli*, 35.2 per cent; *Endolimax nana*, 30.5 per cent; *Dientamoeba fragilis*, 3.9 per cent; *Iodamoeba buetschlii*, 1.4 per cent; *Giardia lamblia*, 32.2 per cent; *Chilomastix mesnili*, 5.1 per cent; *Retortamonas intestinalis*, 2.3 per cent; *Trichomonas hominis*, 1.4 per cent; *Ascaris lumbricoides*, 0.6 per cent; *Enterobius vermicularis*, 4.1 per cent; *Trichuris trichiura*, 0.4 per cent; and *Taenia* sp., 1.2 per cent.

## METHODS OF STUDY AND RESULTS

Identification of the macroscopic adult helminths was possible with the unaided eye. For identification of all other parasitic forms, it was necessary to prepare the fecal specimens for microscopic examination. The techniques used and the contribution each made to the total number of parasites recognized are as follows:

1. *Wet Saline Film*.—Films prepared by placing a coverslip over a thin suspension of feces in Ringer's solution possess the advantages of being simple to prepare and of causing no morphologic distortion. In these preparations, furthermore, it is possible to observe the diagnostically characteristic motility of trophozoites and larvae. Their chief disadvantage lies in the fact that cytologic details are poorly outlined.\* Eighty per cent of all parasites found were identified with this technique alone (Table I). Amoebae, however, particularly *Dientamoeba fragilis* and *Iodamoeba buetschlii*, were not well differentiated.

2. *Wet Iodine-Stained Film*.—The substitution of Lugol's iodine in the preparation of wet films brought into stained relief certain distinguishing morphologic characteristics and inclusions. Confusing plant forms, that is, blastocystis and other yeast, hair roots, and plant cells, were easily identified. Starch grains and particles (blue) and glycogen (pink) stained with iodine. The iodine, however, rapidly stopped locomotion and failed to give complete cytologic detail. About 35 per cent of the parasites which had not been identified in the saline film were differentiated in these preparations, and enough additional parasites were recognized to increase the total survey incidence by about 2 per cent.

\*Diaphragm manipulation or partial dark-field illumination outlines some of the cytologic details.

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This survey was made possible by the assistance of Dr. J. H. Clark of the Pathology Department of the Philadelphia General Hospital, and Dr. D. H. Wenrich of the Zoology Laboratory of the University of Pennsylvania. The author, now in Service, made all the stool examinations.

TABLE I  
COMPARATIVE IDENTIFICATION AND INCIDENCE

PARASITE SPECIES	TOTAL SPECIES	RINGER'S SALINE		LUGOL'S IODINE		IRON-ALUM- HEMATOXYLIN		CONCENTRATION			
		NO.	%	NO.	%	NO.	%	DE RIVAS		ZnSO <sub>4</sub>	
								NO.	%	NO.	%
<i>Entamoeba histolytica</i>	41	30	70	35	85	41	100	15	37	5	100
<i>Entamoeba coli</i>	180	138	77	128	71	180	100	180	100	35	100
<i>Endolimax nana</i>	156	140	90	151	96	155	99	53	34	27	85
<i>Dientamoeba fragilis</i>	20	2	10	6	30	20	100	0	00	2	33
<i>Iodamoeba buetschlii</i>	7	0	00	6	86	7	100	0	00	3	75
<i>Giardia lamblia</i>	165	142	86	129	78	165	100	165	100	43	100
<i>Chilomastix mesnili</i>	26	24	92	16	62	25	96	2	8	3	75
<i>Retortomonas intestinalis</i>	12	12	100	4	33	12	100	0	00	0	00
<i>Trichomonas hominis</i>	7	6	86	3	43	7	100	0	00	0	00
<i>Ascaris lumbricoides</i>	3	2	67	2	67	2	67	3	100	3	100
<i>Enterobius vermicularis</i>	21	21	100	12	57	5	24	0	00	2	33
<i>Trichuris trichiura</i>	2	1	50	1	50	1	50	2	100	2	100
<i>Taenis</i> sp.	6	6	100	3	50	2	33	2	33	2	33
Total	646	524	80	496	76	622	96	422	65	127	93

The column head No. refers to the number of parasites definitely identified by this procedure; this is represented under the column head % as per cent of total.

The iron-alum-hematoxylin slide was used to verify the identification of other films. The less than 4 per cent not identified by this slide were definitely identified on other films but could not be found on the iron-alum-hematoxylin-stained slide (concentrate or film). The actual incidence on the iron-alum-hematoxylin without concentrate material was between 70 and 75 per cent. Nine of the twenty-one *Enterobius* diagnoses were made by recovery of the adult worms from the stool.

3. *Dry-Stained Slide*.—Because of the importance of nuclear cytology in identifying species of amoebae, a routine iron-alum-hematoxylin-stained slide was made on every stool and studied for at least fifteen minutes. The slide was prepared according to the method of Wenrich.<sup>2</sup>

Examination of the stained film made it possible to identify 96 per cent of all observed parasites and to distinguish other morphologically similar organisms and objects. It also served as a semipermanent exhibit of the organism and preserved the material for future comparison and study. On the other hand, it increased the total surveyed incidence by only 1 to 2 per cent.

4. *Concentrated Preparations*.—The de Rivas centrifugation method<sup>3</sup> was used routinely. Concentration by the zinc sulfate centrifugal flotation method was frequently done for comparative study.

The de Rivas centrifugation method concentrated the cysts of *Entamoeba coli* and *Giardia lamblia* and the ova of *Ascaris lumbricoides* and *Trichuris trichiura* very well, but there was some morphologic distortion. The other parasitic forms were concentrated poorly or not at all. Blastocystis and detritus were advantageously removed. The zinc sulfate centrifugal flotation was much less selective and concentrated all surveyed parasites about equally well.<sup>4</sup> The combined concentration methods added 11 per cent to the total number of parasites found in the survey.

#### DISCUSSION

The combined concentration methods permitted the finding and recognizing of the greatest number of parasites; the Ringer's saline wet film was next in this respect. The addition of Lugol's iodine to wet films killed the trophozoites but increased the number of cysts which could be identified. Preparations stained with iron-alum-hematoxylin were valuable chiefly because they provided morphologic details which aided the identification. The number of organisms

recognized on these films was unduly high (Table I) because concentrated material was stained to help the identification of cysts.

The large species of *Entamoeba histolytica* could often be identified in the simple wet films. The mixed amoebic infections and the smaller species of amoeba, however, frequently required iron-alum-hematoxylin stains for differentiation. A large per cent of *Entamoeba coli* could be identified on any of the films; iron-alum-hematoxylin was necessary in some atypical forms and mixed infections. *Endolimax nana* gave little diagnostic difficulty in the wet films except when there was mixed amoebic infection. This was also true of *Dientamoeba fragilis* and *Iodamoeba buetschlii*. *Giardia* and *Chilomastix* were easily identified in any of the films. *Trichomonas hominis* was infrequently confused with the trophozoites of *Chilomastix*. All ova found were easily identified in all films.

#### SUMMARY

1. Eighty per cent of all surveyed parasites were identified by a wet saline film; 35 per cent of the remainder were specifically diagnosed by the wet iodine-stained film. The iron-alum-hematoxylin-stained slide is an invaluable adjunct to differential diagnosis of enterozoic protozoa.

2. Routine concentration contributed 11 per cent to the total surveyed incidence.

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## THE LOW TOXICITY IN ANIMALS OF BORIC ACID AS A PRESERVATIVE AGENT

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PRIOR to 1906 in the United States and to 1928 in England, boric acid was used as a food preservative at levels as high as 0.5 per cent. Wiley,<sup>1</sup> in his classic studies in 1904, showed that symptoms of chronic toxicity appeared in human beings when amounts of from 1 to 5 Gm. of boric acid daily were ingested with food. One-half gram daily, however, was tolerated according to Wiley for fifty days with little or no evidence of ill effect. He concluded, "On the whole, the results show that 0.5 Gm. of boric acid per day is too much for the normal man to receive regularly. On the other hand, it is evident that the normal man can receive this amount for a limited period of time without much danger of impairment of health."

Despite its checkered career as a food preservative, boric acid has continued in general use as a mild antiseptic. Some knowledge of its acute toxicity has appeared in the literature due to its accidental misuse. In 1928 McNally and Rust<sup>2</sup> reported deaths in six infants who were given by mistake saturated solutions containing from 3 to 6 Gm. boric acid. Peyton and Green,<sup>3</sup> in 1941, reported severe toxic effects but survival in a patient who received 700 c.c. of a 4 per cent solution of boric acid by hypodermoclysis. These authors suggest that the toxicity of boric acid in such large doses may be due to a displacement of chloride with a resulting upset of the chloride balance. A fall in blood chlorides during injection of boric acid was offered in support of this theory. Absence of aftereffects anywhere in the patient after receiving an almost fatal dose of boric acid was taken as evidence that the primary toxicity of the drug is functional. In 1943 Ross and Conway<sup>4</sup> reported death of a hospitalized boy following irrigation of an empyema cavity with saturated boric acid solution. The amount of boric acid used was heroic by any standards, about 12 liters in seventy-two hours, a total of about a pound of boric acid.

In early studies Rost<sup>5</sup> and Wiley<sup>6</sup> showed that doses of 3 Gm. of boric acid and upward were rapidly excreted in the urine to the extent of from 82 to 100 per cent. Excretion in feces and sweat was found to be small and quantitatively unimportant. Presnell and Brill,<sup>7</sup> in 1937, further demonstrated the rapid urinary excretion of boric acid.

Kent and McCance<sup>8</sup> called attention to the significant amount of boron which is an inevitable component of our daily diets. Boron is essential to the normal growth and development of several plant species and is now accepted as a constituent of all vegetable tissues. Hove and co-workers<sup>9</sup> found boron in all samples of milk and eggs tested, and Dodd<sup>10</sup> found about 0.005 per cent boric acid in several fresh fruits, with a maximum of 0.109 per cent on a dry basis.

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In balance studies, Kent and McCance<sup>8</sup> showed that a normal turnover from food sources was about 100 mg. of boron per week in two individuals, equal to about 80 mg. of boric acid per day. Added boric acid was quantitatively excreted in a few days.

Kahlenberg<sup>11</sup> and Kahlenberg and Barwesser<sup>12</sup> discovered the truly remarkable fact that boric acid is readily absorbed through intact human skin and may be detected in the urine within fifty-five seconds after immersion of the feet in saturated boric acid solutions. Ochsner<sup>13</sup> had previously shown that mere application to the skin of large compresses saturated with boric acid solution resulted in concentrations of boric acid in the urine of a few hundredths to as much as 0.1 per cent. All tissue membranes tested were readily permeable to boric acid, and this would account for its rapid excretion through the kidney.

We became interested in the toxicity of boric acid when Frost<sup>14</sup> found that riboflavin-boron complexes are considerably more soluble in water than is riboflavin alone. This led further to an investigation of the preservative properties of various levels of boric acid for injectable solutions containing riboflavin alone and in combination with other B complex factors. When it was found that such solutions were well preserved and well tolerated, the use of boric acid as a preservative at 0.5 per cent was extended to other experimental solutions used routinely in animal work in small amounts. Boric acid, unlike other preservative agents, such as the phenols, chlorobutanol, and benzyl alcohol, does not appear to enter into the metabolism of the animal and is excreted quantitatively as such. From this standpoint and because boron is a natural constituent of the diet, it would appear to deserve reconsideration as a preservative agent.

The following experiments are presented as evidence for the essential nontoxicity of low levels of boric acid. The preservative properties of boric acid were also investigated.

*Injection Experiments With Dogs and Rats.*—Long-term nutritional experiments with rats and dogs which received three weekly injections of a B complex preparation containing 0.5 per cent boric acid as a preservative were carried out as follows:

Five puppies started at from 6 to 8 weeks of age on a highly synthetic diet were supplied five B complex vitamins exclusively by injection. The injectable B complex preparation contained the following per cubic centimeter: thiamine, 1 mg.; riboflavin, 1 mg.; nicotinamide, 35 mg.; calcium pantothenate, 2 mg.; pyridoxine, 0.6 mg.; and boric acid, 5 mg. Injections were made thrice weekly at the rate of 0.23 c.c. per kilogram body weight equal to 1.15 mg. boric acid. Thus a 10 kg. dog received 11.5 mg boric acid per injection equal to 0.5 mg. boric acid per kilogram body weight per day. Two puppies, which were both littermates of dogs receiving the B complex preparation with boric acid by injection, received an identical diet with all synthetic B complex factors added to the diet. Since these two dogs did not receive boric acid at any time, they served as controls.

Excellent tolerance of continued intramuscular injection of the above preparation over periods of from twelve to eighteen months was observed. No irritation or itching was observed to occur at the point of injection as is often seen when phenol is used as the preservative. The upper leg muscles of the two hind

legs of the dogs were injected alternately with no attempt to prepare or clean the point of injection. The dogs very soon came to take the injections as a matter of course and even welcomed the attention. There was no outward difference in the growth performance or general behavior between the injected dogs receiving boric acid and those which received their nutritional requirements entirely by mouth with no boric acid.

Similar experiments extending through two generations were carried out with rats on purified diets. Fourteen rats, seven males and seven females, were placed at weaning on a standard purified diet based on sucrose, casein, and salt mixture. These rats received by injection five synthetic B complex vitamins in the form of a preparation as described for the dog experiments. Injections were made at the rate of 0.07 c.c. per rat three times per week. This entailed an average weekly injection per rat of 1.05 mg. boric acid, equal to 0.15 mg. per day. Since the weight of the rats increased from an average of about 45 Gm. at the start of the experiment to about 217 Gm. after about fifty days, the per kilogram injection of boric ranged downward from about 3.3 to 0.7 mg. per kilogram body weight per day.

A control group of twenty-eight rats was fed an identical diet simultaneously but received all B complex vitamins orally as additions to the diet. Rats of both groups were maintained on the respective dietary regimens described through two generations. Matings were made when the rats were 75 days of age. The rate of growth, reproductive performance, average number of offspring in litters, and survival of young were about equal for the two groups as shown in Table I.

TABLE I

GROWTH AND REPRODUCTIVE PERFORMANCE OF RATS ON HIGHLY PURIFIED DIET RECEIVING BORIC ACID BY INJECTION (FROM 0.7 TO 3.3 MG. PER KILOGRAM)

GENERATION	NUMBER OF ANIMALS	AV. WT. 75TH DAY	NUMBER OF LITTERS	AV. NUM- BER IN LITTERS	% YOUNG SURVIVED TO 21ST DAY	AV. WT. 21ST DAY
<i>Group Received Boric Acid by Injection</i>						
		♀      ♂				
Parent	28 (15 ♀)	187   292	13	7	64	37
First	31 (23 ♀)	170   266	13	8	21	32
<i>Control Group</i>						
Parent	14 ( 7 ♀)	175   264	7	7	57	31
First	12 ( 8 ♀)	189   275	6	9	39	28

The differences in per cent survival of the young born to parent and first generation females in the two groups are not considered significant. The same applies to the differences of the average weight at the twenty-first day.

*Subacute Toxicity Studies.*—The object of the following experiments was to study the effects in rats and dogs of injection of large amounts of a solution containing 1.5 per cent boric acid. The solution also contained 0.1 per cent riboflavin.

*Experiments in Rats:* Two groups of six weanling male rats (average weights, 49 Gm.) were injected subcutaneously daily with 2 c.c. of the solution mentioned in the preceding paragraph and 2 c.c. of physiologic saline, respectively. The injections were continued for thirty days and weights were taken

at weekly intervals. The average weight increments of the groups were 106 Gm. and 108 Gm., respectively. The rate of injection of boric acid thus decreased from about 600 mg. per kilogram body weight at the start to about 180 mg. per kilogram at the end.

At the end of the period, urine tests showed the absence of sugar and a trace of albumin for the experimental animals. Blood studies made at the end of the period on three test rats and two control rats revealed no significant differences in hemoglobin, erythrocyte, or differential white blood cell counts.

Autopsies on the test rats showed no gross pathology and no irritation at the sites of injection. Hearts, adrenals, and testicles were normal. The liver showed a moderate degree of cloudy swelling and some fatty infiltration but no necrosis. The kidneys were not different in appearance from the kidneys of untreated controls. No necrosis or glomerular changes were noted.

*B. Experiments With Dogs:* Three adult dogs were injected daily intravenously with 20 c.c. of the test solution. The dogs weighed 6, 6, and 8 kg., respectively, and thus received 50, 50, and 38 mg. of boric acid per kilogram body weight, equivalent to an intake of from 2.7 to 3.5 Gm. of boric acid in terms of a 70 kg. man. Injections were continued daily for thirty days, during which time the weights of the dogs did not change significantly.

If the injections were made rapidly, the dogs became unsteady; however, they always recovered completely within a few moments and otherwise showed a normal behavior throughout the experimental period. With repetition of injections, the equilibrium disturbances disappeared.

Blood values for each dog were determined at the beginning, during, and at the end of the experiment, so that the animals acted as their own controls. No significant changes in hemoglobin, erythrocyte, or differential counts were observed during the course of the experiment.

Two of the dogs were killed one day after the last injection. The urine of both dogs showed an alkaline reaction and was negative for sugar, albumin, and urobilinogen. There was no gross pathology. Histologic examination indicated a normal condition of the adrenals, spleens, hearts, intestines, and lungs. The liver showed a moderate degree of cloudy swelling. Fat stains were negative in both animals. The kidney of one dog showed some amorphous exudate in the glomerular space and in the tubules. There were some old degenerative changes in a few glomeruli. The picture was altogether not considered as presenting a severe degree of renal damage. The kidney of the second dog showed only a mild hyperemia and cloudy swelling.

*Effects on Blood Pressure.*—Two injections of 10 c.c. each of a 1.5 per cent boric acid solution were made intravenously into a chicken under phenobarbital anesthesia. Following each injection, there was a gradual rise of the blood pressure of about 20 mm. Hg, lasting for about three minutes. Injection of an equal volume of saline failed to cause a rise.

Injection of 20 c.c. of the solution in an anesthetized dog of 11 kg. weight, given within three minutes, had no significant effect on the blood pressure.

*Effect on Estrus Cycle.*—The estrus cycles of six female rats were followed for two weeks; then the rats were injected subcutaneously daily at the rate of 12 mg. of boric acid per day for twenty-one days.



Two rats showed a slightly prolonged period of diestrus at first during the injections, but the normal cycles reappeared. The other rats showed no variation from the normal during the injections.

Puhlman,<sup>15</sup> in 1939, reported that 0.5 per cent boric acid in the diet inhibited the estrus cycles of rats and that 1.0 Gm. per kilogram subcutaneously daily caused a picture of incomplete permanent estrus. Such doses are, of course, excessively high and are well above the range which Wiley<sup>1</sup> had previously reported to produce acute toxic symptoms in human beings. Our results show, on the other hand, that daily doses as high as from 60 to 70 mg. boric acid per kilogram body weight in rats does not alter normal estrus when given for twenty-one days.

*Effect of Low Chloride Diets on Toxicity of Boric Acid.*—The interesting suggestion made by Peyton and Green<sup>3</sup> that displacement of chloride might play a role in the toxicity of boric acid was investigated as follows:

Three groups of eight adult rats (from 170 to 220 Gm.) were formed. The rats in Group I were kept on Purina dog chow, those in Group II on a purified diet containing Wesson salt mixture, and those in Group III on a similar purified diet in which the chlorides of the Wesson salt mixture were replaced by sodium and potassium carbonates. All the rats were placed on the diets for one week prior to daily intraperitoneal injections of 4 c.c. of 4 per cent boric acid solution.

In the case of the rats in Group I, no deaths occurred during the three weeks of the experiment and no consistent changes in weights of the animals were noted.

The animals in Group II all soon became listless and died in from five to seven days. In Group III death of the animals occurred in from nine to sixteen days. Thus absence of chlorides did not increase susceptibility of rats to the toxic effects of boric acid. Blood chloride determinations were made on representative animals in Groups II and III. No lowering of blood chlorides was noted during the period of the experiment.

The excellent tolerance to boric acid of adult rats maintained on a normal stock diet was not explained but is conceivably due to the greater nutritive value of this type of ration over the purified diet. Whether the difference is a qualitative or quantitative one was not determined.

*Preservative Properties of Boric Acid for Injectable Solutions.*—The bacteriostatic effectiveness of boric acid was studied at levels of 0.15, 0.3, 0.5 and 1.5 per cent in solutions containing various B complex vitamins. Such solutions were shown to support growth readily in the absence of a preservative. Standard inocula (about 5,000 per cubic centimeter) of *Staphylococcus aureus*, *Escherichia coli*, and mixed mold spores were made in all cases. The samples were kept at room temperature. Colony counts were made from standard platings at regular intervals.

Levels of 0.3 and 0.5 per cent boric acid were effective in causing disappearance of inocula from B complex injectable solutions made to pH 4.5 and 5. Disappearance of bacteria was complete in two days; of mold, in twenty-eight days.

In the case of a solution containing 1.5 per cent boric acid and 0.1 per cent riboflavin buffered at pH 6.5, complete disappearance of viable bacteria required six days; of mold spores, thirty-five days. A comparison was made in this experiment against a riboflavin solution containing 0.5 per cent chlorobutanol as preservative. Although the chlorobutanol caused rapid disappearance of both bacterial strains, presence of mold spores was detected to the thirteenth day. A similar solution containing 0.15 per cent boric acid showed gradual but complete loss of bacterial inocula and considerable diminution of mold spores.

#### DISCUSSION

On the basis of its known toxicity, boric acid compares favorably with such commonly used preservatives for injectable solutions as phenol, the cresols, chlorobutanol, and benzyl alcohol. Furthermore, boric acid possesses the following advantages: low cost, high purity of U.S.P. crystals, lack of color or odor, chemical stability and inertness, and ease of solution. On the physiologic side it has the important advantages of being nonirritating to tissues and of being excreted rapidly in unchanged form.

The fact that boron is a normal food constituent and is an essential growth element for many plant species argues against its cumulative toxicity at very low levels of intake. The maximum tolerance level named by Wiley<sup>1</sup> of 0.5 Gm. per day for human beings is only six times the level reported by Kent and McCance<sup>2</sup> to represent a normal daily turnover from food sources. It seems likely that boric acid exerts toxic effects only when the renal threshold is exceeded and accumulation occurs in the tissues. In all reports of acute toxicity in human beings, relatively massive doses of boric acid have been involved and there is a notable lack of reported cases of chronic toxicity. Thus the chronic cumulative type of toxicity caused by continued ingestion of minute amounts of compounds of lead, mercury, selenium, and fluorine, for example, is apparently not shared by compounds of boron, even though boric has a cumulative action in larger doses, that is, 0.5 Gm. daily.

The easy availability and very widespread use of boric acid by both members of the medical profession and laymen has led to its occasional fatal misuse. Extreme rapidity of assimilation through all membranes of the body, a fact which in many cases appears to have escaped attention, has also led to its misuse.

#### SUMMARY

Boric acid at from 0.5 to 1.5 per cent in injectable solutions showed no toxic effects in rats and dogs when injected over long periods. Levels averaging about 400 mg. per kilogram body weight per day for thirty days did not affect the blood picture or deter the growth of rats. Levels of from 60 to 70 mg. per kilogram appeared to have no effect on the estrus cycle.

Varying the level of chlorides in the diet did not appear to influence the toxicity of injected boric acid; however, it appears likely that other nutritional factors play a part in tolerance for this drug.

The bacteriostatic effect of boric acid, from 0.5 to 1.5 per cent, against injected bacteria and molds appeared satisfactory for preservation of certain injectable solutions.

The bacteriologic work and the long-term nutritional experiments were carried out by Dr. H. W. Cromwell and F. Peirce Dann, to whom we are indebted.

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# RELATIONSHIPS OF CHEMICAL CONSTITUTION TO THE ANTIBACTERIAL EFFECTS OF DERIVATIVES OF 9-AMINOACRIDINE

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THE antibacterial effects of various acridine compounds, in vitro, have been presented in two previous reports from these laboratories.<sup>1, 2</sup> Of significant interest were the comparatively low concentrations of 9-aminoacridine required to exhibit bacteriostatic and bactericidal properties against a variety of organisms commonly associated with wound infections. A 3-chloro-7-methoxy-derivative of 9-aminoacridine proved to have even greater antibacterial effects than the parent compound, particularly against members of the anaerobic clostridium group. It seemed desirable, therefore, to study additional derivatives of 9-aminoacridine and especially those containing the 3-chloro-7-methoxy groups. The compounds tested, together with their molecular weights, are as follows:

1. 3-methyl-7-methoxy-9-(1-methyl-4-diethylamino)butylaminoacridine dihydrochloride (molecular weight, 452.5)
2. 3-chloro-7-methoxy-9-(2-phenyl-4-dimethylaminobutylamino)acridine dihydrochloride (molecular weight, 506.5)
3. 3-chloro-7-methoxy-9-(2-phenyl-4-diethylaminobutylamino)acridine dihydrochloride (molecular weight, 534.5)
4. 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino)butylaminoacridine tartrate (molecular weight, 549)\*
5. 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino)butylaminoacridine formate (molecular weight, 491)\*
6. 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino)butylaminoacridine citrate (molecular weight, 591)\*
7. 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino)butylaminoacridine acetylsalicylate (molecular weight, 759)\*
8. 3-chloro-7-methoxy-9-[(1-methyl-4-phenylpiperidyl-4)methyl]amino acridine dihydrochloride (molecular weight, 536.5)
9. 3-chloro-7-methoxy-9-(4-dimethylamino)phenylaminoacridine dihydrochloride (molecular weight, 451)
10. 3-chloro-7-methoxy-9-[(1-benzyl-4-phenylpiperidyl-4)methyl]amino acridine dihydrochloride (molecular weight, 594.5)
11. 3-chloro-7-methoxy-9-(6'-methoxy-8'-quinolylamino)acridine dihydrochloride (molecular weight, 489)
12. 3-chloro-7-methoxy-9-(3-pyridylamino)acridine dihydrochloride (molecular weight, 409)

## METHODS

The methods which were used in the two previous reports were followed in this investigation. This consisted essentially of preparing a series of dilutions

\*Salts of Quinacrine, U. S. P.

From the Research Laboratories, Winthrop Chemical Co., Inc

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TABLE I  
HIGHEST DILUTION OF COMPOUND SHOWING ANTIBACTERIAL ACTIVITY

COMPOUND	PNEUMOCOCCUS						STREPTOCOCCUS					
	TYPE I		TYPE II		TYPE III		HEMOLYTIC		VIRIDANS		AGALACTIAE	
	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
1	256,000	256,000	512,000	512,000	256,000	256,000	256,000	32,000	64,000	8,000	64,000	16,000
2	256,000	256,000	512,000	512,000	256,000	256,000	128,000	64,000	64,000	4,000	64,000	32,000
3	256,000	128,000	1,024,000	256,000	16,000	16,000	128,000	64,000	128,000	8,000	256,000	128,000
4	256,000	128,000	512,000	256,000	128,000	128,000	128,000	64,000	32,000	16,000	32,000	32,000
5	128,000	128,000	256,000	128,000	128,000	128,000	128,000	64,000	32,000	16,000	64,000	32,000
6	128,000	128,000	256,000	128,000	128,000	128,000	128,000	64,000	32,000	16,000	32,000	32,000
7	128,000	32,000	256,000	128,000	128,000	128,000	128,000	16,000	16,000	8,000	32,000	16,000
8	128,000	64,000	256,000	128,000	128,000	64,000	256,000	32,000	64,000	32,000	32,000	1,000
9	1,000	<1,000	<1,000	<1,000	1,000	<1,000	1,000	<1,000	1,000	<1,000	1,000	<1,000
10	<1,000	<1,000	1,000	<1,000	1,000	<1,000	1,000	<1,000	<1,000	<1,000	<1,000	<1,000
11	1,000	<1,000	2,000	<1,000	1,000	<1,000	2,000	<1,000	<1,000	<1,000	<1,000	<1,000
12	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000

Bs = Bacteriostatic.

Bc = Bactericidal.

&lt;1,000 = Concentrations greater than 1:1,000 not tested.

TABLE II  
HIGHEST DILUTION OF COMPOUND SHOWING ANTIBACTERIAL ACTIVITY

COMPOUND	STAPHYLOCOCCUS AUREUS		BACILLUS YANEUS		WELCHII		TETANI		HISTOLYTICUS		OEDEMATENS	
	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
	CLOSTRIDIUM											
1	2,000	<1,000	<1,000	64,000	128,000	32,000	128,000	1,000	128,000	1,000	128,000	4,000
2	16,000	2,000	<1,000	16,000	16,000	8,000	16,000	8,000	32,000	1,000	32,000	4,000
3	16,000	2,000	<1,000	32,000	32,000	8,000	32,000	8,000	32,000	1,000	32,000	4,000
4	4,000	<1,000	<1,000	8,000	8,000	2,000	8,000	2,000	16,000	<1,000	16,000	1,000
5	4,000	1,000	<1,000	32,000	32,000	8,000	32,000	8,000	32,000	1,000	32,000	2,000
6	4,000	1,000	<1,000	16,000	8,000	4,000	8,000	4,000	8,000	2,000	8,000	1,000
7	4,000	<1,000	<1,000	8,000	8,000	2,000	16,000	2,000	8,000	4,000	8,000	<1,000
8	8,000	1,000	<1,000	16,000	16,000	8,000	16,000	8,000	16,000	1,000	16,000	4,000
9	<1,000	<1,000	<1,000	16,000	4,000	32,000	32,000	32,000	32,000	32,000	16,000	4,000
10	<1,000	<1,000	<1,000	1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000
11	1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000
12	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000

Bs = Bacteriostatic.

Bc = Bactericidal.

&lt;1,000 = Concentrations greater than 1:1,000 not tested.

of each compound in an appropriate broth medium. After autoclaving the drug-broth solution, each dilution series together with a drug-free control, was inoculated with a loopful of a twenty-four-hour broth culture of one of the test organisms. The test organisms included types I, II, and III pneumococci, beta hemolytic streptococcus No. C-203, *Streptococcus viridans*, *Streptococcus agalactiae*, *Staphylococcus aureus* No. 209, *Bacillus pyocyaneus*, *Clostridium welchii*, *Clostridium tetani*, *Clostridium histolyticus*, and *Clostridium oedematiens*.

The inoculated tubes were incubated at 37° C. and examined for visible growth after twenty-four, forty-eight, and seventy-two hours. Failure of growth to appear in twenty-four hours was considered to be evidence of bacteriostasis. Tubes which failed to show growth after seventy-two hours were tested for the presence of living organisms by transferring three loopfuls to fresh medium lacking the drug. Lack of growth in the subculture tube was assumed to be evidence of a bactericidal effect on the part of the drug in the original drug-broth mixture. The results of these studies are given in Tables I and II.

#### RESULTS AND DISCUSSION

From data presented in Tables I and II, we note that compounds 1 through 8 are active against most of the bacteria tested. Compounds 9 through 12, however, are essentially devoid of any antibacterial activity. The acetylsalicylate, citrate, tartrate, and formate salts of quinaerine (compounds 4 through 7) displayed an antibacterial activity comparable to quinaerine itself. From the results obtained with compounds 9, 11, and 12, it would appear that substitution in the 9- position with an aromatic amine tends to decrease the activity of the compound markedly. By comparing compounds 8 and 10, we note that antibacterial effects are markedly increased by replacing the N-benzyl group (compound 10) with a methyl group (compound 8).

#### CONCLUSION

The activity of 3-chloro-7-methoxy-9-aminoacridine on bacteria is not increased by replacement of the amino group by certain N-containing alkyl or aryl amines. This conclusion is based upon in vitro studies of twelve such derivatives tested against various organisms which may be associated with wound infections.

The authors wish to express appreciation to Dr. C. E. Kwartler and Mr. P. Lucas for the preparation of some of the compounds. The methods of preparing the compounds will be presented elsewhere. Acknowledgment is also given to Virginia L. Wilson for technical assistance in the bacteriologic study.

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## LABORATORY METHODS

### A FRACTIONAL SUPPLEMENTARY TEST FOR DISTINGUISHING POSITIVE KAHN REACTIONS IN SYPHILIS AND MALARIA

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**F**ALSE reactions in serologic tests for syphilis resulting from malaria have been recognized by many workers. With troops returning to civilian life from malarious districts, the problem becomes more acute not only because of the stigma attached to the "positive" serologic report, but also for diagnostic reasons. The expected number of false positive reactions obtained with the standard Kahn test on malarial individuals is as high as 47.5 per cent at some stages of the disease.<sup>1</sup>

The fact that several diseases are known to be responsible for false reactions has brought about investigation of the so-called confirmatory tests, the purpose of which is to verify the fact that syphilis alone is responsible for the positive reaction. Kahn<sup>2-4</sup> has published details of three such techniques, the basis of the tests being, respectively, (1) differential temperature, (2) differential electrolyte concentration, and (3) salt dispersibility. Chargin and Rein<sup>5</sup> commented favorably on the first method as a result of their studies. Discrepancies were observed in some blood specimens subjected to repeated "verification tests." De Groat,<sup>6</sup> after submitting malarial bloods to Kahn's laboratory for the differential temperature verification test, believes the test valuable in this disease. No discussions of the other two methods have been found to date. Rytz<sup>7</sup> also has published a differential method which removes from serum all but traces of protein by the use of a weak copper sulfate solution. Upon the deproteinized substance is performed the Rytz flocculation test. No data concerning malaria were presented.

I<sup>1</sup> observed that with serums of malarial individuals a constant pattern of reactivity is found when several standard serologic tests are performed. The fact that the Eagle flocculation test and the Hinton test are usually negative when the Kahn test is either doubtful or positive suggested that there existed an actual difference in the character of the immunologic substances causing flocculation in syphilis as contrasted with those in malaria. Since the standard Kahn test is used so extensively, and also because it exhibits a high incidence of false positives in malaria, it was made the basis of a new confirmatory procedure. The serum rather than the other factors was modified.

The possibility was considered that the flocculating substance induced by the malaria and the syphilitic "reagin" might be associated with different serum protein fractions. Therefore precipitation of malarial and syphilitic sera, both



of which gave positive Kahn reactions, was carried out employing various quantities of saturated ammonium sulfate. The several fractions thus obtained were each tested by the Kahn standard test. It was found that syphilitic reagin was attached to the first fraction brought down, whereas the malarial antibody was not so fixed. In other fractions, however, both antibodies were present in varying quantity. Upon this point then is based the following supplementary test which differentiates between a true syphilitic blood and a blood with a positive reaction induced by malaria. This procedure is advantageous in that it can easily be performed in laboratories equipped to do the standard Kahn test.

#### METHOD

A saturated solution of ammonium sulfate as used in the Kahn test on spinal fluids is required. No other special apparatus or solutions are essential.

The standard Kahn test is first performed on all sera to determine the degree of positivity. Only specimens free of hemolysis should be employed.

To 1 c.c. of inactivated serum in a Kahn tube at room temperature is added exactly 0.35 c.c. saturated ammonium sulfate solution. It is necessary to dry the outside of the pipette used to measure the saturated solution prior to its addition in order to insure that no excess is added. A 1 c.c. serologic pipette graduated in hundredths is used for this purpose. No crystalline ammonium sulfate should be pipetted over. The tube is tapped to mix the substances thoroughly and allowed to stand for, and not more than, two minutes, after which the tube is centrifuged at from 1,000 to 2,000 r.p.m. for three minutes (keeping switch in the "on" position for three minutes). Failure to comply strictly with the quantity and the time stated will result in the precipitation of serum fractions which will yield false positive results.

Following the centrifugation the tube is inverted to discard the supernatant fluid and while still in the inverted position is allowed to drain on filter paper for one minute. The precipitate will adhere to the bottom of the tube.

Physiologic saline (0.9 per cent) is then added to the precipitate, the quantity depending on the original Kahn test reading as follows:

If the reading was doubtful ( $\pm$  or 1+), add 0.7 c.c. saline.

If the reading was positive (2+ or 3+), add 0.9 c.c. saline.

If the reading was positive (4+), add 1.2 c.c. saline.

Negatives (see under Discussion) may be tested by assuming that the serum gave a "doubtful" result.

After several minutes, assisted by gentle tapping, the precipitate goes completely into solution. The fractionation is now completed and the testing of this fraction is carried out by performing a Kahn test in the standard manner. However, only the third tube of the three-tube test is employed, using 0.0125 c.c. antigen emulsion plus 0.15 c.c. of the solution containing the dissolved precipitate. The tube is shaken in a Kahn shaker for three minutes, 0.5 c.c. saline is added, and the tube reading is made immediately. Standard Kahn test readings of plus-minus, 1 plus, or 2 plus are given a "negative" report, whereas a 3 plus or 4 plus is given a "positive" report. A "positive" in this sense is interpreted to mean that the fraction carried syphilitic reagin; a "negative," that no reagin was originally present.

## DISCUSSION

The results obtained in this laboratory are presented in Table I. Although the number of cases of malaria of necessity is omitted, the percentage values are based on all malarial patients giving false positive serologic results which occurred over several months. The diagnosis of malaria was confirmed in all instances by positive blood smears, and serologic "malarial patterns" were obtained.

TABLE I

NUMBER OF CASES	DIAGNOSIS	STANDARD KAHN TEST POSITIVE AND DOUBTFUL (PER CENT)	FRACTIONAL SUPPLEMENTARY TEST POSITIVE (PER CENT)
*	Malaria	100	3.2
9	Syphilis (untreated)	100	100
50	Syphilis (treated)	100	90

\*Number of cases deleted in accordance with censorship regulations.

Qualitative factors were more important than quantitative factors as far as the precipitation was concerned. This became evident when quantitative Kahn tests were done on all positives herein reported. Of the group of malarial sera, there were thirty-four with titers of 40 Kahn units or more which gave "negative" supplementary results while of the syphilitic sera there were nine "positives" with titers of less than 40 Kahn units, proving that isolation of the fraction definitely removes the malarial nonspecific-reacting substances.

It is of interest that in the treated patients with syphilis, 10 per cent of the sera were not positive in the supplementary test. This is evidence that the fraction usually carrying reagin may fail to do so in treated syphilis. The titer of reagin is again not determinative inasmuch as the sera of six untreated patients with syphilis with titers of 4 Kahn units were positive, whereas the sera of the five treated patients giving "negative" supplementary results had titers of 4 Kahn units or more. It is suggested, if this observation is confirmed and amplified, that it may be used as an index of complete cure in those patients who remain seroresistant after supposedly adequate treatment has been concluded. Serologic tests usually have been approved on the basis of specificity and sensitivity. It seems to be as important to evaluate tests on the basis of "persistence" also, which sets forth the concept that a continued positive in a completely treated and cured patient with syphilis is as false as any positive serologic reaction obtained from unrelated diseases. It should be noted that Rytz<sup>7</sup> reported of his differential method that sera of patients with syphilis treated for more than a year and a half gave a negative confirmatory reaction although routine serologic tests were positive.

As a finding of interest, mention should be made of two syphilitic bloods which gave negative results in the standard Kahn test and positive results in the Hinton tests. Upon removal of the fractions used in the supplementary test, these two serums gave "positive" results. Where the standard Kahn failed to demonstrate a positive to begin with, reagin was nevertheless demonstrable after the first fraction was removed from the remainder of the serum under the conditions detailed for the present test. The failure of the reagin to be evident in the original serum may be due to the same phenomenon of inhibition

described by Brown.<sup>8</sup> In the present instance the inhibiting substance or substances were presumably removed. This finding also is in need of further consideration.

Patients with diseases other than malaria that produce false serologic reactions were not available at this installation so that the efficacy of this fractional supplementary test in other conditions is not known. It is logical to assume that if the linkage of antibody is the same as in malaria, the same results can be expected. These determinations remain to be carried out.

It is obvious that the fractionation is crude in that total protein and albumin-globulin ratios may vary from individual to individual.

#### CONCLUSIONS

1. A supplementary test for syphilis is described based on the isolation and testing of a fraction of the serum which carries with it syphilitic reagin and only rarely the immunologic substance or substances induced by an infection with malaria.

2. The fractional supplementary test is practical, easily performed, although demanding of accuracy in technique, and can be utilized by any laboratory routinely doing the standard Kahn test.

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# COMPARATIVE RESULTS OBTAINED WITH THE KAHN STANDARD AND A ONE-TUBE FLOCCULATION PROCEDURE IN SEROLOGIC TESTS FOR SYPHILIS

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FOR the past four years advantage has been taken of the availability of the blood specimens submitted in the annual U. S. Public Health Service Evaluation of Serodiagnostic Tests for Syphilis to determine the sensitivity and specificity of a single-tube diagnostic flocculation procedure as compared with the Kahn standard three-tube technique as carried out by Kahn and as performed in our own laboratory. Kahn standard antigen was used in all three procedures, but in the single-tube technique the proportion of patient's serum to antigen was 10.1, that is, 0.2 c.c. serum to 0.02 c.c. antigen. Otherwise, the technique of the author-serologist was followed meticulously, with the tests being read by artificial light and a mirror and the two readings, made at a fifteen-minute interval, averaged. In reporting the results of the one-tube test, readings averaging 2 plus or more were called positive; if the average was 1 plus, the test was called doubtful; and if the average was plus-minus ( $\pm$ ) or negative, the report was negative.

The sensitivity and specificity ratings were worked out in Dr. Mahoney's office at the Venereal Disease Research Laboratory of the U. S. Public Health Service.

In Table I, *A*, it is indicated that 495 specimens from presumably normal, or at least nonsyphilitic individuals, were tested with no false positive or doubtful tests reported by Kahn or our own laboratory, using the Kahn standard test. One false positive and no false doubtful result was obtained with the one-tube method.

In Table I, *B*, are given the results in 156 cases of early syphilis. Of these, Kahn found 110 positive and we found 112 with the three-tube test. With the one-tube test, 111 were positive.

In Table I, *C*, are shown the findings in 371 cases of syphilis in the latent stage. Kahn detected 265 and we detected 246 with the standard test, whereas 262 were positive with the one-tube technique.

In Table I, *D*, are included 304 cases of late syphilis. Kahn found 258 and we found 239 positive with the Kahn standard procedure, while with the one-tube test we detected 250.

In Table I, *E*, are shown the results on sera from 43 cases of congenital syphilis. In this group Kahn detected 36 and we detected 30 with the Kahn standard test. With the one-tube method we picked up 34.

In Table I, *F*, are summarized the findings in 874 sera from syphilitic patients. Of these, Kahn found 669 and we reported 627 positive with the three-

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tube technique, while with the one-tube test we found 657 positives. In *F* it is also indicated that, in our hands at least, significantly fewer doubtful tests were reported with the one-tube than with the three-tube test.

TABLE I

RESULTS OBTAINED IN THE LABORATORY OF DR. R. L. KAHN AND THE ALABAMA STATE DEPARTMENT OF HEALTH USING THE KAHN STANDARD FLOCCULATION TEST AND A ONE-TUBE TECHNIQUE (SPECIMENS SUBMITTED IN THE U. S. PUBLIC HEALTH SERVICE EVALUATION OF SERODIAGNOSTIC TESTS FOR SYPHILIS, 1941, 1942, 1943, 1944)

SURVEY	SPECI- MENS	KAHN STANDARD			ALA. KAHN STANDARD			ALA. ONE-TUBE		
		POS.	DOUBT.	NEG.	POS.	DOUBT.	NEG.	POS.	DOUBT.	NEG.
<i>Nonsyphilitic</i>										
<i>A</i>	495			495			495	1		494
<i>Early Syphilis</i>										
<i>B</i>	156	110	9	37	112	5	39	111	7	38
<i>Latent Syphilis</i>										
<i>C</i>	371	265	16	90	246	22	103	262	12	97
<i>Late Syphilis</i>										
<i>D</i>	304	258	12	34	239	19	46	250	13	41
<i>Congenital Syphilis</i>										
<i>E</i>	43	36	1	6	30	4	9	34		9
<i>Syphilitic (Summary)</i>										
<i>F</i>	874	669	38	167	627	50	197	659	32	185

In Table II is given the comparative standing as to sensitivity and specificity of the three-tube and one-tube tests for four years in the U. S. Public Health Service Annual Evaluation Study.

TABLE II

RESULTS OBTAINED IN THE LABORATORY OF DR. R. L. KAHN AND THE ALABAMA STATE DEPARTMENT OF HEALTH USING THE KAHN STANDARD FLOCCULATION TEST AND A ONE-TUBE TECHNIQUE (SPECIMENS SUBMITTED IN THE U. S. PUBLIC HEALTH SERVICE EVALUATION OF SERODIAGNOSIS TESTS FOR SYPHILIS, 1941, 1942, 1943, 1944)

YEAR		SENSITIVITY	SPECIFICITY
1941	Dr. Kahn standard	79.2	100.0
	Alabama standard	67.0	100.0
	Alabama one-tube	72.2	100.0
1942	Kahn standard	80.7	100.0
	Alabama standard	75.5	100.0
	Alabama one-tube	78.3	100.0
1943	Kahn standard	71.8	100.0
	Alabama standard	70.9	100.0
	Alabama one-tube	71.7	100.0
1944	Kahn standard	82.1	100.0
	Alabama standard	84.7	100.0
	Alabama one-tube	83.6	99.4

## CONCLUSION

A one-tube test for syphilis, using patient's serum and Kahn standard antigen in a 10:1 ratio, is essentially as sensitive and as specific as the three-tube Kahn standard technique, has the advantage of giving fewer doubtful reactions, and is simpler and easier to perform.

# AN IMPROVED METHOD FOR DETERMINING THE COAGULASE ACTIVITY OF STAPHYLOCOCCI BY MEANS OF THE PLASMA AGAR PLATE

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THE observation of Loeb<sup>1</sup> that certain strains of staphylococci were able to coagulate goose plasma was followed a few years later by the demonstration of the relationship of that property of the staphylococci to their pathogenicity.<sup>2</sup> Through the studies of numerous other workers,<sup>3-5</sup> coagulase production has been accepted as a means of ascertaining the possible importance in disease production of staphylococci isolated from human sources. In many clinical laboratories this test has become a routine procedure, either replacing or supplementing the determination of hemolytic activity and mannite fermentation.

The usual procedure for determining coagulase production is a test tube method in which the staphylococci are inoculated into the diluted plasma. The tubes are incubated at 37° C. and read at the end of three and eighteen hours. While this procedure is not laborious, it does require that sterile plasma be kept on hand and that the dilutions be prepared each time a determination is made. Furthermore, the organism must be isolated in pure culture, which entails a delay in reporting results.

In order to simplify the procedure used for coagulase determinations, it occurred to us that coagulase formation might possibly be determined in solid media to which plasma had been added. This would make possible the storage of plasma agar plates to be used as needed and also make possible a more rapid determination by the streaking of such plates directly with those specimens in which staphylococci had been observed in the direct smear preparations.

In the preliminary study, sterile plasma was added to equal amounts of previously sterilized and cooled veal infusion 4 per cent agar. This was well mixed by rotation and 20 c.c. amounts poured into sterile Petri dishes and allowed to solidify. The plates were inoculated heavily in scattered spots with twenty-four-hour cultures of staphylococci which had previously been tested for coagulase production by the test tube method. After overnight incubation at 37° C., there appeared around each colony known to be coagulase positive a gray halo of variable diameter. No zone appeared around the noncoagulase-producing staphylococcus strains. It was further noted that staphylococci growing on plates prepared in a similar manner, but using serum obtained from clotted blood instead of plasma, did not produce this halo effect but only a faint clear zone around certain of the strains. This eliminated the possibility that some substance other than coagulase was giving this halo reaction.

The gray halo in the plasma agar plates first appeared within three or four hours, at which time the majority of cultures could be read. In a few cases the

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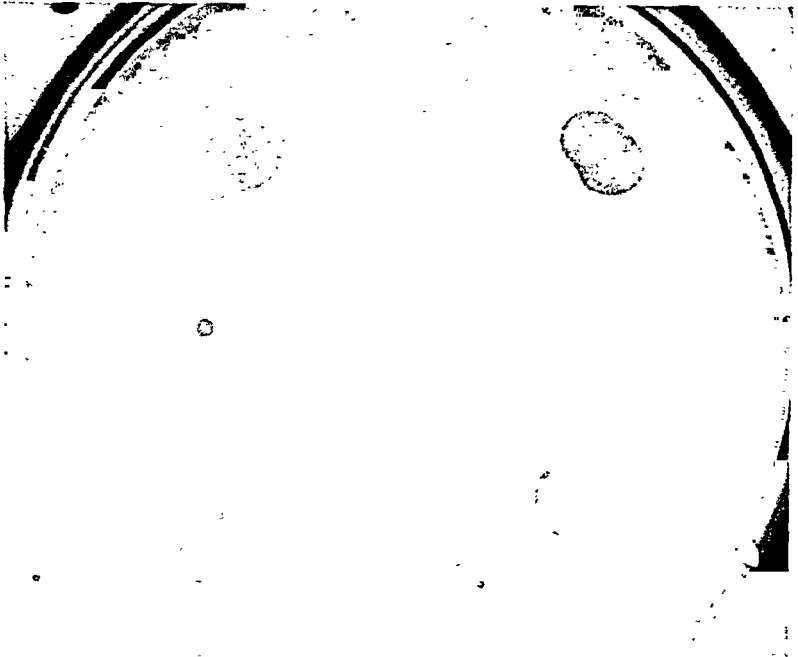


Fig. 1.—Plasma agar inoculated with coagulase-positive and coagulase-negative staphylococci. The production of a gray halo about the coagulase-positive strains differentiates them from the noncoagulase-producing staphylococci; eighteen-hour incubation at 37° C.

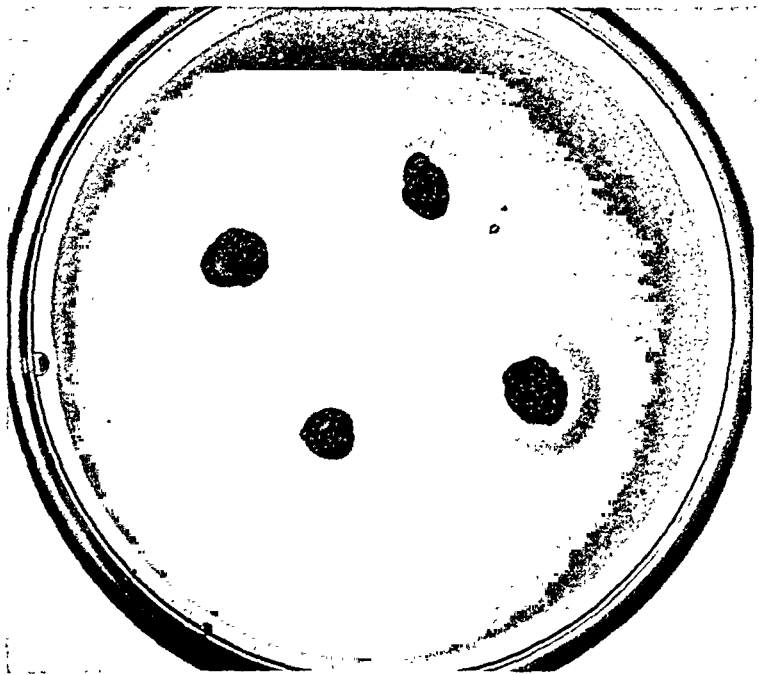


Fig. 2.—Plasma agar inoculated with staphylococci to show a second type of reaction that may occur with certain coagulase-positive strains. Note the area of clearing that is produced within the gray halo as contrasted with the reactions obtained in Fig. 1; eighteen-hour incubation at 37° C.

halo was not visible until the second reading at the end of eighteen hours. After overnight incubation, a zone of clearing was noted around many of those strains which were hemolytic on blood agar plates. Since it did not appear uniformly around all colonies which were known to be hemolytic, it could not be used as a criterion of hemolytic activity. However, nonhemolytic strains did not give this reaction. This clear zone did not interfere with the reading of the zone of coagulase formation but seemed to have pushed it outward so that the final appearance was a clear zone adjacent to the colony, about the periphery of which was a gray zone of variable diameter indicating coagulase activity. The various types of staphylococcus activity on plasma agar plates are shown in Figs. 1 and 2.

Prepared plates were stored for as long as thirty days and the reaction still occurred when these plates were inoculated with coagulase-positive staphylococci, thus indicating that deterioration in storage is not a great problem. Plates not over two weeks old, however, give the best results.

Various concentrations of plasma and types of media were used before obtaining one that we considered most suitable for routine use.

#### MATERIALS AND METHODS

*Plasma Agar.*—Veal infusion 2 per cent agar, containing 0.5 per cent peptone and 0.5 per cent sodium chloride, is prepared in the usual manner. This agar is sterilized and cooled to 43° C. As a fairly clear infusion agar is essential for easy reading of the reaction, the melted agar is poured into a sterile flask, leaving behind the sediment that may have accumulated. To this is added 20 per cent human plasma. We have made use of the plasma discarded by the blood bank because of positive serologic reactions. The agar is then poured into sterile Petri dishes in from 12 c.c. to 15 c.c. amounts and allowed to solidify. Prepared plates are stored in the icebox until ready for use.

Numerous strains of staphylococci can be tested for coagulase production on a single plate. Our usual procedure has been to divide the plate into sections and heavily inoculate an area approximately 4 mm. in diameter in each section with a staphylococcus growth obtained from solid media. The streak method of inoculation is also satisfactory, although the gray zone may be smaller due to the smaller size of the colonies. Incubation is carried out at 37° C. Reactions are recorded at the end of three hours and again after overnight incubation.

Where it is desirable to obtain an early report on the coagulase activity of staphylococci seen in the initial smears of infectious material received in the laboratory, direct plating of this material can be made on plasma agar plates and readings made the following morning. The streaking of the plate should be carried out in such a manner as to obtain isolated colonies. An example of this method of procedure is shown in Fig. 3.

*Tube Method.*—A loopful of culture from a twenty-four-hour veal infusion agar slant was inoculated into 1 c.c. of a 1:3 dilution of plasma. The tubes were incubated at 37° C. and read at the end of three and eighteen hours.

*Hemolytic Activity.*—Hemolytic activity was determined by inoculating the staphylococci from a twenty-four-hour veal infusion agar slant onto veal infusion



5 per cent sheep blood agar plates. The plates were incubated at 37° C. for twenty-four hours before reading.

*Mannite Fermentation.*—This was determined by the inoculation of mannite broth from twenty-four-hour cultures of the staphylococci grown on a veal infusion agar slant. The tubes were incubated at 37° C. and initial readings were made at the end of twenty-four hours with a final reading at the end of one week.

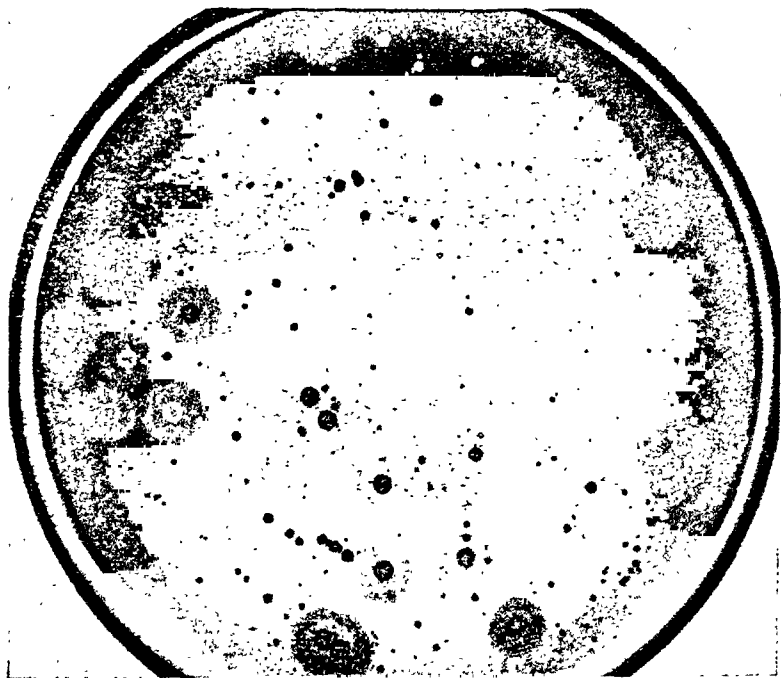


Fig. 3.—Plasma agar inoculated by streak method with sputum specimen containing coagulase-positive staphylococci; eighteen-hour incubation at 37° C.

#### RESULTS

Eighty-two strains of staphylococci, of which sixty-three were *Staphylococcus aureus*, seventeen *Staphylococcus albus*, and two *Staphylococcus citreus*, were used in this study. A comparative study of their ability to coagulate human plasma by the tube method and by the plate method described was made. The results are indicated in Table I together with such activities as mannite fermentation and hemolytic activity on blood agar plates.

Sixty-two of the *Staphylococcus aureus* strains were obtained from human sources and clinically were definitely related to the pathologic process from which they were isolated. One strain in this group was a stock strain used for testing the potency of penicillin preparations. All of these strains gave a positive coagulase reaction in eighteen hours by the tube and plate methods. It is to be noted, however, that in the three-hour reading of coagulase activity, only fifty-four of the strains gave a positive reaction by the tube method in this period of time. This was probably due to the fact that the plates are inoculated heavily in a small area, hence the beginning coagulase activity with certain strains is more quickly noted on the plate than in the tube, where the

TABLE I

RESULTS OBTAINED WITH EIGHTY-TWO STRAINS OF STAPHYLOCOCCI IN COMPARING THE VALUE OF THE TUBE AND PLATE COAGULASE TEST (MANNITE FERMENTATION AND HEMOLYTIC ACTIVITY ALSO SHOWN)

SPECIES	NO. TESTED	MANNITE (NO. POS.)	HEMOLYTIC (NO. POS.)	TUBE COAG. (NO. POS.)		PLATE COAG. (NO. POS.)		SOURCE
				3 HR.	18 HR.	3 HR.	18 HR.	
<i>Staph. aureus</i>	63	62	59	54	63	59	63	Definite pathologic process
<i>Staph. albus</i>	17	4	3	1	0	0	0	Various sources not related to pathologic process
<i>Staph. citreus</i>	2	0	0	0	0	0	0	

organisms are dispersed throughout the diluted plasma. The ability of this group of organisms to ferment mannite and to hemolyze red blood cells coincided well with coagulase production, although a few discrepancies in this respect can be noted in Table I.

The seventeen strains of *Staphylococcus albus* were from various sources which could not be correlated with an infectious process. None of them gave a positive coagulase test. As will be noted in Table I, there was some irregularity in the correlation between the activity of this group in mannite and on blood agar plates, although as would be expected in coagulase-negative strains, the majority of them were inactive in mannite and did not produce hemolysis on blood agar plates.

The two strains of *Staphylococcus citreus* were isolated from contaminated plates and were negative in all tests.

## SUMMARY

The ability of certain strains of staphylococci to produce coagulase is a recognized means of determining their possible pathogenicity. This property is determined by inoculation of the staphylococci into tubes of diluted plasma. The tubes are then incubated at 37° C. and read for plasma clot formation at intervals of time, usually from three to eighteen hours. This procedure has the disadvantages of requiring sterile plasma to be kept on hand and dilution tubes to be prepared, as well as necessitating the isolation of the organism before the test can be made.

In order to simplify this procedure and to obtain rapid determinations for clinical laboratory reporting, we have developed a modified test which gives readings comparable with those obtained by the "standard" method. By the use of veal infusion agar plates to which 20 per cent sterile plasma has been added, many of the disadvantages of the tube method of coagulase determination can be obviated. Coagulase-producing staphylococci inoculated onto such plates produce a gray halo effect about the inoculum within three to eighteen hours. Noncoagulase-producing staphylococci do not give a reaction. Numerous strains of staphylococci can be tested on a single plate. Plates of this medium can be prepared and stored for use as needed, eliminating the necessity of preparing dilution tubes each time a test is run.

For clinical laboratory purposes where it is desirable to obtain an early reading on the coagulase-producing power of a staphylococcus seen in smears of infectious material, this material can be streaked directly on plasma agar

plates. After overnight incubation of these plates the coagulase-producing activity of the organism can be determined by observing single isolated colonies. This eliminates the necessity of isolating the organism before the determination is made. Infectious material in which numerous other organisms besides staphylococci are present can be handled in a similar manner as long as isolated colonies of staphylococci are obtained, since we have not observed any interference by other organisms with this reaction.

As this media supports the growth of many of the pathogens such as streptococci, pneumococci, and meningococci, it may also be found useful as routine culture media for clinical laboratories.

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## SIMPLE TECHNIQUE FOR ESTIMATION OF PENICILLIN IN BLOOD AND OTHER BODY FLUIDS\*

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WITH THE TECHNICAL ASSISTANCE OF MARY W. CUTTING

THE Oxford cup method for the assay of penicillin, while applicable to fluids containing fairly high concentrations of the drug, is unsatisfactory for clinical determinations in the blood where the concentration is usually less than 1 unit per cubic centimeter. The method of Rammelkamp<sup>1</sup> is more commonly used for estimations in the blood but is laborious. Fleming's method, as described to us by Herrell,<sup>2</sup> of the Mayo Clinic, is simpler, and it is this procedure which has been further simplified by us. The three methods are basically similar. Each consists of serial dilution of the unknown blood and addition of a constant inoculum of hemolytic streptococci. After incubation the tubes are observed for evidence of inhibition of growth of the bacteria and compared with controls, similarly prepared, but containing known amounts of penicillin. The penicillin content of blood from man, dog, and rabbit, containing variously from 0.01 to 4 units per cubic centimeter, was repeatedly compared by the three methods with satisfactory agreement.

### METHOD (BLOOD)

*Unknown.*—Set up eight small test tubes (Wassermann) in a suitable rack. To each, except the first, add 0.2 c.c. of physiologic salt solution. To the first and second tubes add 0.2 c.c. of the unknown whole (citrated) blood. From the second tube transfer 0.2 c.c. to the third tube, then from the third tube transfer 0.2 c.c. to the fourth, and so on, to make serial dilutions. Discard 0.2 c.c. from the final tube.

To each of the eight tubes add 0.2 c.c. of a suspension of hemolytic streptococci in broth prepared as follows: Transfer streptococci from a twenty-four hour culture in broth, by loop, to fresh broth. Use one loop of streptococci for each cubic centimeter of broth, and prepare enough for all the unknown and control blood samples to be assayed.

Shake each tube, hold almost horizontally, and from it, by capillarity, nearly fill a glass capillary tube about 6 cm. long. While still holding the capillary tube horizontally, imbed the end in a layer of modeling clay, filling a groove in a small board. When all the capillary tubes are in place, in a row, turn the board so that the tubes are upright.

Incubate for twenty-four hours at 37° C., and then inspect the tubes for presence of hemolysis and colonies of streptococci.

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†Richards' strain or other fast-growing strain.

From the Department of Pharmacology and Therapeutics, Stanford University School of Medicine.

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*Control.*—Set up a series of tubes, as for the unknown blood, but use defibrinated horse blood containing 1 unit of penicillin per cubic centimeter in place of the unknown blood sample.

*Estimation.*—The most dilute control capillary tube without hemolysis and bacterial colonies indicates the dilution of 1 unit of penicillin which will inhibit the inoculum of streptococci. The preceding tube indicates the dilution of 0.5 units which will inhibit the streptococci and so forth. By comparison of the end point in the control series and that in the unknown series, the concentration of penicillin in the latter is estimated. Thus, if the end point in the control series is in the sixth tube, as it usually is, the unknown contains 1 unit, if it, also, gives the end point in the sixth tube; 0.5, if in the fifth tube, and so forth.

#### COMMENT

Sterilized glassware is used, but the tubes and pipettes are not flamed, except initially. To simplify preparation, the test tubes (Wassermann) are placed, without plugging, in a large wire rack, which is then wrapped in paper and sterilized. A small tear in one corner allows removal of individual tubes as needed. Any capillary tubes may be used, but in our hands the Kimble brand, No. 34500, for melting point determinations, has been most satisfactory.

Although the concentration of red cells differs in each tube, this does not affect the accuracy of the test. In this procedure the centrifuging of blood specimens and the addition of known amounts of red cells are eliminated. The simplification of the procedure, therefore, depends upon these omissions and upon the use of capillary tubes. Rabbit blood occasionally gives results in which the end point is difficult to determine, presumably because of delay in sedimentation of the red cells. Because penicillin is stated to enter red blood corpuscles in minute amounts only, any considerable shift in hematocrit would affect the concentration of penicillin when determined in whole blood, as in our procedure. However, the error seldom is significant, especially when considered in the light of the large inherent error of the serial dilutions.

The concentration of penicillin in other body fluids may be determined similarly. Urine must be diluted before estimation to bring the usually high urinary penicillin levels into the range of blood levels. It is usually diluted with nine parts of defibrinated horse blood, but broth may be used (the end point is then determined only on the basis of colony growth). The final values must, of course, be corrected for the dilution factor.

The age of the streptococci (up to five days), their dilution between  $10^0$  and  $10^{-4}$ , and the exact period over which the test is allowed to run have practically no effect upon the results obtained.

#### CONCLUSION

A simplification of Fleming's method for the estimation of penicillin is described.

#### REFERENCES

1. Rammelkamp, C. H.: A Method for Determining the Concentration of Penicillin in Body Fluids and Exudates, *Proc. Soc. Exper. Biol. & Med.* 51: 95, 1942.
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## COMPARISON OF METHODS ADAPTABLE TO PRODUCTION LINE EXAMINATION OF SPUTUM FOR TUBERCLE BACILLI

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FOR several years there has been a need for some method of digestion and concentration of tubercle bacilli in sputum as a routine procedure in the Division of Laboratories of the Tennessee Department of Public Health. Some of the factors considered desirable were: (1) a simple method of concentration adaptable to use on many specimens; (2) sufficient increase in positive findings to make the procedure worth while; (3) elimination of extraneous material which might interfere with the identification of a few organisms in the microscopic field.

Several methods were available which might prove satisfactory. Accordingly, a series of examinations was made to determine which of these methods should be employed routinely. Preliminary checks had been made by Brougher and one of us,<sup>1</sup> comparing the direct smear with the autoclave (heat coagulation) method and the trypsin digestion method. The autoclave method was found more efficient in these studies than the trypsin method. This fact, together with the safety and simplicity of the autoclave method, allowed its choice in preference to the trypsin digestion for further study.

Robinson and Stovall (1941)<sup>2</sup> compared NaOH digestion and concentration with a number of other methods listed as follows:

1. Alkalies, other than NaOH, as calcium hydroxide and ammonium hydroxide
2. Acids (6 per cent sulfuric)
3. Animal ferments (trypsin)
4. Organic solvents ("tergitol penetrant 08" in solution with NaOH)
5. Vegetable ferments (caroid)

These authors make the following statement in regard to these methods: "From the study made it appears that the most satisfactory digesters are normal sodium hydroxide and caroid (2 to 5 per cent caroid with 0.2 to 0.1 per cent sodium hydroxide)." The use of caroid (commercial preparation of the enzyme papain), as presented by Sullivan and Sears (1939),<sup>3</sup> was not tried in this comparison of methods of concentration. Robinson and Stovall state: "Caroid, while it does not digest pus actively, is more satisfactory than trypsin." They suggest equal quantities of 0.1 to 0.2 per cent sodium hydroxide solution and a 5 or 2 per cent caroid solution as being more effective than either alone. They state further that caroid smears usually have an even background and good distribution of well-stained organisms, although decolorization is occasionally difficult. The advantages of this method, as described by the authors, are as follows: (1) rapid digestion; (2) no neutralization of digestant necessary; (3)

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*Control.*—Set up a series of tubes, as for the unknown blood, but use defibrinated horse blood containing 1 unit of penicillin per cubic centimeter in place of the unknown blood sample.

*Estimation.*—The most dilute control capillary tube without hemolysis and bacterial colonies indicates the dilution of 1 unit of penicillin which will inhibit the inoculum of streptococci. The preceding tube indicates the dilution of 0.5 units which will inhibit the streptococci and so forth. By comparison of the end point in the control series and that in the unknown series, the concentration of penicillin in the latter is estimated. Thus, if the end point in the control series is in the sixth tube, as it usually is, the unknown contains 1 unit, if it, also, gives the end point in the sixth tube; 0.5, if in the fifth tube, and so forth.

#### COMMENT

Sterilized glassware is used, but the tubes and pipettes are not flamed, except initially. To simplify preparation, the test tubes (Wassermann) are placed, without plugging, in a large wire rack, which is then wrapped in paper and sterilized. A small tear in one corner allows removal of individual tubes as needed. Any capillary tubes may be used, but in our hands the Kimble brand, No. 34500, for melting point determinations, has been most satisfactory.

Although the concentration of red cells differs in each tube, this does not affect the accuracy of the test. In this procedure the centrifuging of blood specimens and the addition of known amounts of red cells are eliminated. The simplification of the procedure, therefore, depends upon these omissions and upon the use of capillary tubes. Rabbit blood occasionally gives results in which the end point is difficult to determine, presumably because of delay in sedimentation of the red cells. Because penicillin is stated to enter red blood corpuscles in minute amounts only, any considerable shift in hematocrit would affect the concentration of penicillin when determined in whole blood, as in our procedure. However, the error seldom is significant, especially when considered in the light of the large inherent error of the serial dilutions.

The concentration of penicillin in other body fluids may be determined similarly. Urine must be diluted before estimation to bring the usually high urinary penicillin levels into the range of blood levels. It is usually diluted with nine parts of defibrinated horse blood, but broth may be used (the end point is then determined only on the basis of colony growth). The final values must, of course, be corrected for the dilution factor.

The age of the streptococci (up to five days), their dilution between  $10^0$  and  $10^{-4}$ , and the exact period over which the test is allowed to run have practically no effect upon the results obtained.

#### CONCLUSION

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for from one to three hours had no ill effects. Digestion was aided by shaking or stirring the specimens at least once during incubation. Following incubation, 1 to 2 drops of phenol red indicator were added to each specimen. Sufficient 3 per cent HCl was added to neutralize the specimens. Then, a few more drops were added until the reaction was definitely acid. In practically all cases a good flocculation was obtained.

The sputa in the third series of tubes were used for clorox digestion and concentration. To this series equal volumes of clorox were added. The specimens were stirred well with applicators, allowed to stand from ten to thirty minutes, and stirred well again before they were centrifuged.

Timing was arranged so that the three series of tubes were ready for the centrifuge at the same time. The specimens were centrifuged at 3,000 r.p.m. for thirty minutes.

Three sets of new, chemically clean slides were used. Smears were made from the centrifuged sediment after the supernatant was discarded. An applicator, prepared with a wisp of cotton, was used to make the smears. After air drying and fixation by heat, all slides were stained by the Ziehl-Neelsen method.

Slides were placed on a rack, carbolfuchsin added, and the preparations steamed for five minutes. After cooling for ten minutes, they were washed with tap water and decolorized with acid alcohol. They were then washed again and counterstained with Loeffler's methylene blue, diluted 1:4 with distilled water. Undiluted Loeffler's was found to give too deep staining for all organisms to show up well and the diluted counterstain gave sufficient background for contrast.

Specimens found negative by routine direct smear and positive by concentration method were rechecked by direct examination. Except where otherwise specified, observations include the recheck.

#### OBSERVATIONS

Two hundred and eleven specimens were examined. Of these, seventy-five were found to contain tubercle bacilli while 136 were negative. The seventy-five sputa showing tubercle bacilli were collected in fifty-one instances for purposes of diagnosis and in sixteen as a check on treatment; for the remaining eight, no reason for collection was stated.

TABLE I  
BREAKDOWN COMPARISON OF SEVENTY-FIVE POSITIVE FINDINGS

NUMBER	DIRECT	CLOROX	NaOH	AUTOCLAVE
1	+	+	+	-
4	-	-	-	+
9	-	+	-	-
2	-	+	+	-
4	-	+	-	+
45	+	+	+	+
9	-	+	+	+
1	+	-	-	-

Table I shows that one specimen was found positive by only the direct method, four by only the autoclave, and nine by only the clorox method. The other sixty-two specimens were found positive by two or more methods.



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# A RAPID STAINING TECHNIQUE FOR ACID-FAST ORGANISMS

CORPORAL HUGH E. MULLER AND STAFF SERGEANT RALPH L. CHERMOCK

**R**APID routine staining of acid-fast organisms with carbolfuchsin usually employs heat. Heat serves to produce more rapid penetration of the stain.

We have recently employed Tergitol No. 7\* a detergent or wetting agent, as a substitute for heat and have found that it also accelerates the staining of acid-fast organisms. Staining occurs even more rapidly than with heat. Tergitol No. 7 was chosen because one of us had previously employed it successfully in histologic technique.<sup>2</sup>

## A. TECHNIQUE FOR STAINING SMEARS

The following technique was applied to smears which included fresh sputum containing tubercle bacilli, concentrations of sputum prepared by the Petroff and phenol methods, and pure cultures of tubercle bacilli.

**Method.**—The smears are fixed with heat and stained for one minute with modified Kinyoun's carbolfuchsin, the formula<sup>1</sup> for which is as follows:

Basic fuchsin .....	4 Gm.
Phenol crystals .....	8 Gm.
95 per cent ethyl alcohol .....	20 c.c.
Distilled water .....	100 c.c.
Tergitol No. 7 .....	1 drop to every 30 to 40 c.c. of above mixture

**Note.**—This stain loses its desired rapid effect over a period of a few days. It is desirable to add the Tergitol No. 7 to the already mixed stain prior to use.

Next the smears are decolorized for one-half minute with acid alcohol, the following formula being used:

HCl .....	3 c.c.
95 per cent ethyl alcohol .....	97 c.c.

They are then counterstained for one minute with Loeffler's alkaline methylene blue, using the following formula:

Potassium hydroxide (10 per cent aq. sol.) ..	0.07 c.c.
Distilled water .....	70.0 c.c.
Stock solution methylene blue .....	30.0 c.c.

**Note.**—Methylene blue stock—1.48 Gm. to 100 c.c. of 95 per cent ethyl alcohol. Potassium hydroxide first added to water to make 1/10,000 dilution, then dye added.

Finally the smears are rinsed with water and dried.

\*Tergitol No. 7 obtained from the Carbide and Carbon Chemical Corporation, New York, N. Y.

<sup>1</sup>Phenol method for tubercle bacilli concentration in sputum: To the sample of sputum to be concentrated, an equal volume of 5 per cent phenol is added. This is mixed well and autoclaved at 15 pounds pressure for fifteen minutes. The specimen is cooled, the upper portion is decanted, and the remainder centrifuged at 1800 r.p.m. for from ten to fifteen minutes. The sediment is streaked on the slide, dried by air, fixed with heat, and stained.

From the Laboratory Service, Army Air Forces Regional Station Hospital No. 1, Coral Gables, Fla.

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This staining method was found to give more consistent results than were obtained with the standard Ziehl-Neelsen method. The acid-fast organisms stained more intensely, were more easily discernible in the field, and the detail of the individual organism was more pronounced. This was especially noticeable with the beaded bacillary form of the tubercle bacillus. Moreover, in certain instances we have been able to demonstrate acid-fast bacilli with the method described in known tuberculous infections when similar preparations were negative where heat was employed instead of Tergitol No. 7. In the staining of smears, the substitution of a 1.5 per cent solution of fast green as a counterstain for Loeffler's alkaline methylene blue was found to be very satisfactory.

Several experiments were performed in an attempt to test the specificity of the method for acid-fast organisms. A hundred sputum smears known to contain no tubercle bacilli were stained by the new technique and examined for possible false staining reactions. No acid-fast organisms were found. The stain was then applied to smears made from pure cultures of a wide variety of the common bacterial genera, and again no false reactions were observed. Sputum smears collected from other laboratories and found to contain tubercle bacilli when stained by the standard methods were also positive in all instances when stained by the new technique.

#### B. TECHNIQUE FOR STAINING TISSUE SECTIONS

The use of the new modified stain was applied to sections of tissue known to be infected with *Mycobacterium tuberculosis*. The staining process was again accelerated and excellent differentiation of the organisms was obtained after five minutes' staining. One difficulty encountered, however, was the selection of a counterstain which would give morphologic detail and sharp contrast with the organisms. Loeffler's alkaline methylene blue gave poor results and little contrast. Heidenhain's hematoxylin differentiated the tissues adequately, but the similarity of color to the acid-fast stain made it difficult to distinguish the organisms. After testing a variety of stains, fast green, described in the following paragraphs, was selected as the most desirable counterstain. In preparations counterstained for two minutes with fast green, the organisms stood out so clearly against a green background that they could be identified easily under high dry magnification.

*Method.*—Tissues are fixed in formalin for twenty-four hours, embedded in paraffin by any of the standard techniques, sectioned, and mounted on slides. The slides are immersed in the following:

1. Xylol for three minutes
2. Xylol for two minutes
3. 95 per cent ethyl alcohol for two minutes
4. 80 per cent ethyl alcohol for two minutes
5. 70 per cent ethyl alcohol for two minutes
6. 50 per cent ethyl alcohol for two minutes
7. 30 per cent ethyl alcohol for two minutes
8. Distilled water for two minutes and
9. Modified Kinyoun's carbolfuchsin stain, prepared as previously described, for five minutes

They are then rinsed with distilled water, decolorized with acid alcohol until the tissues are light pink in color, and counterstained with fast green for two minutes. The fast green staining solution is prepared by adding 1.5 c.c. of a saturated alcoholic solution of fast green to 98.5 c.c. of distilled water. After the counterstaining, slides are again rinsed with water, immersed in 95 per cent ethyl alcohol for one minute, absolute ethyl alcohol for two minutes, xylol for two minutes, and in a second jar of xylol for five more minutes. Coverslips are applied with balsam or gum damar.

If the hematoxylin-carbolfuchsin method is used, the slides are prepared as through step 8 above. The tissue is then stained with Harris hematoxylin for ten minutes. This time may be decreased to from 15 to 3 minutes by adding 1 drop of Tergitol No. 7 to each 30 to 40 c.c. of the hematoxylin stain. The slides are next rinsed in tap water and destained with acid alcohol prepared by adding 1 c.c. of concentrated hydrochloric acid to 100 c.c. of 75 per cent ethyl alcohol. They are again rinsed in tap water and immersed in ammonia water (2 to 3 drops of ammonium hydroxide in 100 c.c. of distilled water) for five minutes. After a third rinsing in water, they are stained for five minutes with the modified Kinyoun's carbolfuchsin stain, rinsed in water, destained with acid alcohol (concentrated hydrochloric acid, 3 c.c.; 95 per cent ethyl alcohol, 97 c.c.) and rinsed a final time. They are then immersed in 95 per cent alcohol for one minute, absolute alcohol for two minutes, xylol for two minutes, and a second xylol jar for five minutes. Coverslips are applied with balsam or gum damar.

#### CONCLUSION

Rapid staining techniques for acid-fast organisms in smears and tissues are presented. The detergent Tergitol No. 7 was used to replace heat. The shorter time required for staining and the ease with which the procedure can be employed constitute improvements in technique which make the methods particularly applicable to routine use. Equal or better results are obtained with these procedures than with the older methods, and none of the required specificity for acid-fast organisms is lost.

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# GERHARDT'S TEST FOR ACETO-ACETIC ACID IN URINE

## A SIMPLIFIED METHOD FOR DISTINGUISHING BETWEEN TRUE AND FALSE POSITIVE REACTIONS

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**I**N ORDER to distinguish between a positive Gerhardt test due to aceto-acetic (diacetic) acid and one due to certain drugs, for example, acetylsalicylic acid (aspirin), sodium salicylate, phenacetin, phenazone (antipyrine), one of the following tests is usually performed:

1. A fresh portion of the urine is boiled and the test repeated. If the color was due to a drug, the second test will also be positive; if due to aceto-acetic acid, the second test will be negative. As pointed out by Harrison,<sup>1</sup> this method is reliable only if the urine is boiled vigorously in a large boiling tube or in an open vessel for several minutes.

2. The colored solution is boiled for a few minutes. If the color is due to aceto-acetic acid, it will disappear; if due to a drug, it remains.

As Todd and Sanford<sup>2</sup> point out, however, "it is not sufficient to boil the urine after the color has been brought out by the ferric chloride, as is sometimes advised, since the color caused by certain of the drugs will then disappear as well as that caused by diacetic acid."

The following modified technique, the outcome of experiments in this department, is recommended as a routine procedure.

*Gerhardt's Test.*—Add about 4 c.c. (1-inch column) of urine to 2 or 3 c.c. ( $\frac{1}{2}$ -inch column) of 10 per cent ferric chloride solution in a test tube. It should be noted that aceto-acetic acid and all the drugs mentioned, except phenacetin, give an immediate positive reaction. The dark brown color due to phenacetin develops slowly and only reaches its maximum intensity after two or three minutes.

If the test is positive, add 1 drop of concentrated nitric acid to 1 inch of urine in a test tube and boil for about one minute. Cool thoroughly under the tap and add  $\frac{1}{2}$  inch ferric chloride solution.

1. If the urine contains aceto-acetic acid, the second test will be negative.

2. If the urine contains a drug, the second test will be positive; when moderate or small amounts are present, the color may be slightly reduced in intensity.

3. If the urine contains both aceto-acetic acid and a drug, the result obtained will depend on the concentration of the drug. With moderate or small amounts the color will be much less intense in the second test. If large amounts of a drug are present, the color will still be so intense after boiling with nitric acid that any fading of the color due to the disappearance of aceto-acetic acid may not be perceptible.

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# THE CONSTRUCTION, CALIBRATION, AND USE OF AN ALTERNATING CURRENT ELECTRODYNAMIC BRAKE BICYCLE ERGOMETER

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MANY types of ergometers have been employed for the purpose of investigating physiologic problems relating to the working efficiency of the human body. Faulty design, poor construction, and crude computations limited the practical usefulness of many of these devices. Methods of measuring the amount of work done by the arms included lifting weights, shoveling sand and coal, rowing, pumping water, and turning a crank, and various forms of ergometers were used. In problems involving the use of the legs, climbing of stairs, walking on a treadmill, wheeling a loaded wheelbarrow up an incline, and riding a bicycle have been employed extensively.

Recent attempts in this field of research have shown that the bicycle ergometer has many advantages over other methods of measuring work output. The first advantage is in the muscle groups involved in pedaling. The extensor muscles of the legs are the strongest and most highly developed muscles of the body and consequently make possible much higher rates of working, prolonged periods of working, and a greater total output. The second advantage lies in the fact that the head and thorax of the working subject are more or less stationary. This is a distinct advantage in collecting continuous data on respiratory reactions and in making observations relative to physiologic changes during the activity period. Other advantages arise from the inherent features of the machine which make possible controlled variations in the load, constancy in the rate of working, and accurate and automatic recording of data.

Bicycle ergometers which have been constructed by various investigators in the past have been of two types. A friction brake was used in the earliest models. Due to uneven braking action and uncontrollable loss of heat, the friction brake did not prove to be a satisfactory measuring device. The results are grossly inaccurate, and refinement of technique, which might partially overcome these deficiencies, becomes exorbitant in time and expense. Recent models of the bicycle ergometer have utilized the principle of the electrodynamic braking action of the electrical generator. This latter form of bicycle ergometer has been developed and perfected and now provides us with a reliable, flexible, and completely automatic method of measuring the work output and power of the leg muscles.

The earliest investigations in the use of an electric brake bicycle ergometer were made by Atwater and Benedict,<sup>1</sup> who simply pressed the drive wheel of a small dynamo to the rear wheel of a bicycle and measured the current generated. This approach did not prove to be reliable for there was considerable slip in the contact between the drive wheel and the rear wheel of the bicycle.

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This made for uncertainty in the determination of the amount of work done. Furthermore, it was not possible to vary the working load or measure the constancy of the rate of working. Benedict and Carpenter<sup>2</sup> and Benedict and Cady<sup>3</sup> employed an electric brake bicycle ergometer which consisted essentially of a bicycle, the rear wheel of which was replaced by a copper disk. Turning the pedals caused the copper disk to rotate between the pole faces of an electromagnet and provided a constant source of resistance. The resistance or load could be varied by changing the magnitude of the current flowing through the coils of the electromagnet. This ergometer proved to be fairly accurate and reliable when properly used but had the disadvantage of being difficult to calibrate and did not provide a convenient method of recording data on work output. Kelso and Hellebrandt<sup>4</sup> designed a recording electrodynamic brake bicycle ergometer which was completely automatic, employing a direct current generator as the brake. In their ergometer the armature of the generator was connected to the pedal sprocket of the bicycle and the pedaling motion rotated the armature. The field coils of the generator were independently excited by a 12-volt storage battery, producing a magnetic field in which the armature revolved. When the revolving armature was placed in an electric circuit, in this case a resistor and a recording voltmeter in parallel, a current flowed through it. This current set up a magnetic field which opposed the magnetic field of the field coils, thereby producing the braking action of the ergometer. By varying the flow of current in the field coils, the braking action could be controlled accurately. Through careful calibration of the instrument it was then possible to interpret the terminal voltage of the armature in terms of work rate.

The apparatus herein described is essentially the same as that used by the last-mentioned investigators but has the added feature of being operated by alternating current. The current for exciting the field coils is obtained by means of a rectifier unit built into the circuit, thereby providing a reliable and convenient source of direct current.

#### CONSTRUCTION AND CARE OF THE ERGOMETER

In appearance and principle, the ergometer described herein is similar to that designed and constructed by Kelso and Hellebrandt.<sup>4</sup> In addition to the substitution of a rectifier unit to replace the storage battery for providing the field current, changes were made in the type and size of the various parts to make the machine more suitable for the purpose in mind.

*Details of Construction.*—The ergometer is portable, reliable, completely automatic, and of sturdy construction. Fig. 1 shows the over-all arrangement and placement of the various parts. Fig. 2 is a schematic wiring diagram and shows the electrical circuit used.

The framework is constructed of heavy steel girders and piping supported by four-inch, rubber-tired, ball-bearing castors. This construction provides a framework which is strong and rigid but still of high mobility, an important consideration since the ergometer is used in various parts of the laboratory. The seat, handlebars, frame, pedals, sprocket, and chain are parts of a high-grade bicycle. They are attached to the framework so as to simulate an ordinary bicycle. The seat and handlebars are movable to permit adjustments for body build.

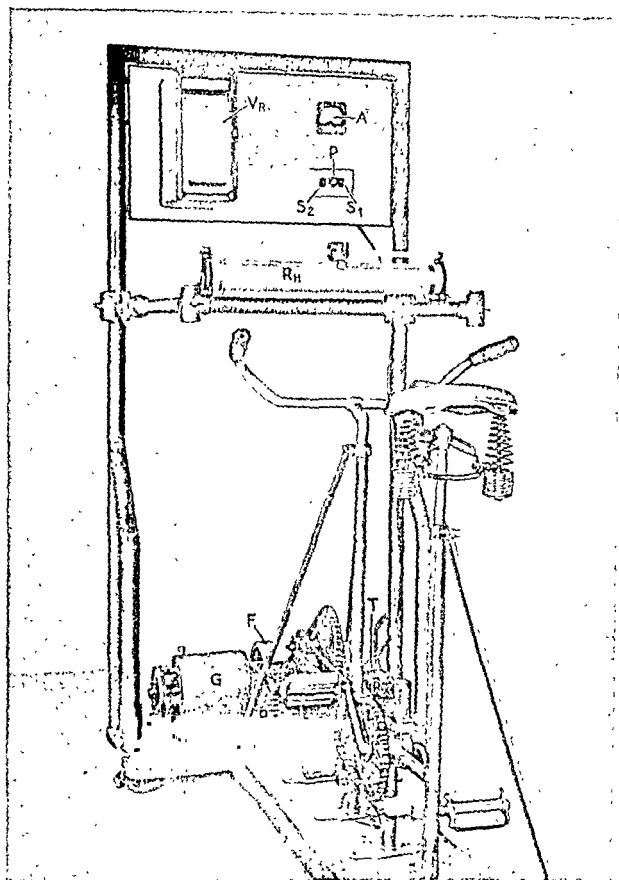


Fig. 1.—Photograph of the bicycle ergometer showing the placement of the main parts.  $V_R$ , Recording voltmeter;  $A$ , ammeter;  $P$ , pilot light;  $S_1$  and  $S_2$ , switches;  $R_h$ , field rheostat;  $G$ , generator;  $F$ , flywheel;  $T$ , step-down transformer;  $R_r$ , load resistor;  $R_r$ , rectifier.

The pedal sprocket of the bicycle is connected to the drive shaft of a 12-volt direct current Dodge automobile starter-generator ( $G$ , Figs. 1 and 2) by means of a double sprocket and chain arrangement which gives a 1:12 ratio between the pedals and the generator armature. One revolution of the pedals, by this arrangement, results in twelve revolutions of the armature. An 8-inch flywheel ( $F$ , Fig. 1), weighing 15 pounds, is fastened to the armature drive shaft to provide rotary inertia for smooth pedaling. The series winding of the generator



is disconnected and is not used. The shunt winding is wired into the circuit so that it is independently excited to provide the magnetic field for the braking action of the generator.

The direct current for exciting the shunt winding, hereafter called the field coils ( $F$ , Fig. 2) of the generator, is obtained from a 110-volt alternating current source by means of a 32-volt step-down transformer and a Mallory magnesium-copper sulfide rectifying element ( $R_x$ , Figs. 1 and 2) in a single phase, full-wave bridge circuit. The rectifier is a type S36B9 with a maximum alternating current operating voltage of 28.7 and an inductive load of 12.8 volts (direct current) and 10.6 amperes (direct current). A 15-ampere direct current ammeter ( $A$ , Figs. 1 and 2) is placed in this circuit to measure the current flowing through the field coils. A 7.7-ohm, 12-ampere rheostat ( $R_h$ , Figs. 1 and 2) is included in the circuit to make adjustments of the field current possible.

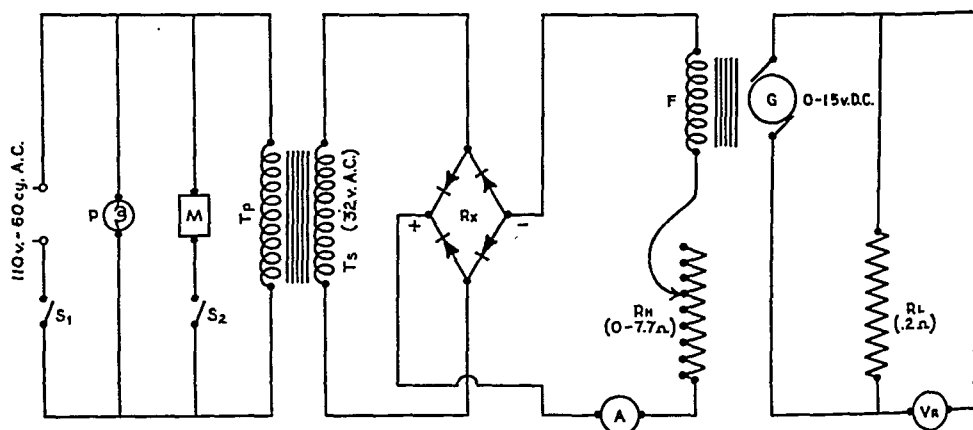


Fig. 2.—Schematic wiring diagram for the bicycle ergometer.  $P$ , Pilot light;  $M$ , motor on chart drive of voltmeter;  $S_1$  and  $S_2$ , switches;  $T_p$ , transformer primary;  $T_s$ , transformer secondary;  $R_x$ , rectifier;  $F$ , generator field;  $G$ , generator armature;  $R_h$ , field rheostat;  $R_l$ , load resistor;  $A$ , ammeter;  $V_r$ , recording voltmeter.

Slight adjustments of this rheostat are needed constantly to meet the fluctuations of the voltage in the alternating current power lines. Larger adjustments can be made to vary the load. Since the braking action of the generator varies directly with the flow of current through the field coils, this arrangement, which is common to all ergometers based upon the electrodynamic braking action of a generator, makes it possible to adjust the load to suit the muscular strength of the subject or to meet experimental requirements and to assure accurate duplication of working conditions at all times.

The terminal brushes of the generator armature are connected to the leads of a fixed resistor ( $R_l$ , Figs. 1 and 2) which has a resistance of .2 ohms. This resistor consists of 140 cm. of 12-gauge constantan wire which has a resistivity of 49 micro-ohm-centimeter. This alloy has a temperature coefficient of resistivity of .00001 and therefore provides a constant resistance over the temperature range under which it operates. A recording voltmeter ( $V_r$ , Figs. 1 and 2) is connected to the leads of the fixed resistor to measure the drop in potential between the brush terminals of the armature. This voltage varies with the rate of pedaling and is therefore a reliable measure of the power output of the genera-

tor. This particular instrument is a 12-volt Westinghouse type GX-40 switch-board recording direct current voltmeter with a chart drive operated by a 115-volt, 60-cycle synchronous motor-driven clock. The chart drive is adjustable for speeds between three-fourths of an inch per hour and three inches per minute. Change gears are conveniently mounted in the chart mechanism and may be changed at will. The motor on the chart drive (*M*, Fig. 2) and a pilot light (*P*, Figs. 1 and 2) are operated directly from the 110-volt alternating current.

Two switches control the operation of the complete apparatus. *S*<sub>1</sub> (Figs. 1 and 2) is in the 110-volt alternating current power line and controls the entire setup. *S*<sub>2</sub> (Figs. 1 and 2) is in the chart drive circuit, making it possible to operate the machine without using the recording feature of the voltmeter.

*Care of the Apparatus.*—The efficiency and reliability of the ergometer depend upon the care exercised in maintaining the parts in good working order. All movable parts must be kept clean and well lubricated. The sliding contact on the rheostat and the armature brushes on the generator must be kept free of dirt and corrosion to prevent excessive electrical resistance at these points. The recording voltmeter must be installed where it will not be subjected to excessive vibration, dust, moisture, or variations in temperature. These conditions are easily met, but care must be taken to avoid the accumulation of dust inside the mechanism.

Under experimental conditions where information as to name of subject, date, time of day, etc., is to be written on the record, it becomes necessary to remove this outer case quite frequently. To avoid this inconvenience and to forestall the possibility of excessive accumulation of dust on the moving parts of the voltmeter, an opening was cut in the glass window which forms the front surface of the case and this opening was filled with a glass door which is easily opened. A small glass window was also placed in the top of the case to permit inspection of the capillary pen. These changes plus proper care have been profitable in the saving of time and in increased efficiency of operation.

*Checking the Apparatus.*—The bicycle ergometer should be checked frequently to guarantee efficiency of operation. A convenient method for determining the accuracy of the recorded voltage is to ride the bicycle at a uniform rate, preferably 60 cycles of the pedals per minute. This rate may be established with the aid of a metronome set to produce 120 clicks per minute. It is recommended that for pedaling at uniform rates 2 clicks per cycle be employed so alternate clicks will coincide with alternate leg movements. This recommendation is made since it was found that when a single click per cycle was used, the subject showed more fatigue in the leg which applied force in unison with the clicks.

When this procedure is followed accurately, the work rate for 60 cycles per minute is 1,500 kg. meters per minute and the generated voltage is 4.4 volts. A work rate of 40 cycles per minute is equivalent to 765 kg. meters per minute and the generated voltage is 3.1 volts. Any uniform rate is satisfactory for checking purposes, but these rates are recommended because they are the easiest to reproduce and they constitute moderate working conditions.

## CALIBRATION TECHNIQUE

Calibrating the bicycle ergometer is simply a problem of determining and separating the power losses at the speeds at which the ergometer is to be used. The mechanical power input to the ergometer can be resolved into three general quantities: (1) the friction losses, (2) the electrical losses due to the resistance of the generator windings, and (3) the electrical power delivered to the external load (resistor  $R_1$ , Fig. 2) of the generator. The power for the field circuit of the generator is supplied from a separate source and therefore need not be considered in this problem. Measurement of electrical power is a simple matter but usually direct determination of mechanical power is rather difficult. The mechanical losses are most conveniently calculated by running the generator as a motor.

*Measurement of Frictional Losses.*—The power input to the generator when it is run as a motor consists of the electrical losses due to the resistance of the armature windings and the mechanical power delivered to the shaft to overcome the friction of the bicycle. By running the machine, with the generator as a motor, at the various speeds at which it is to be operated, the total power input at these speeds can be measured with an ammeter and a voltmeter by the following formula:

$$\text{Power input} = \text{Amperes} \times \text{volts}$$

The first step in determining the electrical losses in the motor is to block the motor so the armature cannot move and to obtain the armature resistance with an ammeter and a voltmeter by the following formula:

$$\text{Resistance} = \frac{\text{Volts}}{\text{Amperes}}$$

In larger machines the resistance of the armature circuit is not constant but appears to drop off as the current increases. However, measurements over a wide range of currents show that this particular machine does not follow this rule but has practically constant resistance. The error involved by neglecting this phenomenon and using an average value of resistance is very slight. The next step is to compute the electrical losses in the motor for all the speeds at which it is tested by the following formula:

$$\text{Power loss} = (\text{amperes})^2 \times \text{resistance of the armature}$$

These losses are subtracted from the total electrical power input at each speed to obtain the frictional or mechanical losses.

*Measurement of Electrical Losses Due to Resistance of the Armature Windings.*—The internal electrical losses of the generator functioning as a motor were computed in the previous step. However, the current produced by the generator, when used normally, greatly exceeds the current required to operate it as a motor under the conditions described. For this reason it is necessary to recompute these values to determine the internal electrical losses under operating conditions. The formula for obtaining these losses is the same as that used in the previous step.

*Measurement of the Electrical Power Delivered to the External Load.*—The power delivered to the external load is equivalent to the electrical power dissipated in the load resistor ( $R_1$ , Fig. 2). It may be computed by the formula given or from the following formula:

$$\text{Power} = \frac{(\text{Volts})^2}{\text{Load resistance}}$$

If the load resistance is not known, it may be computed by following the procedure employed in the case of the armature resistance.

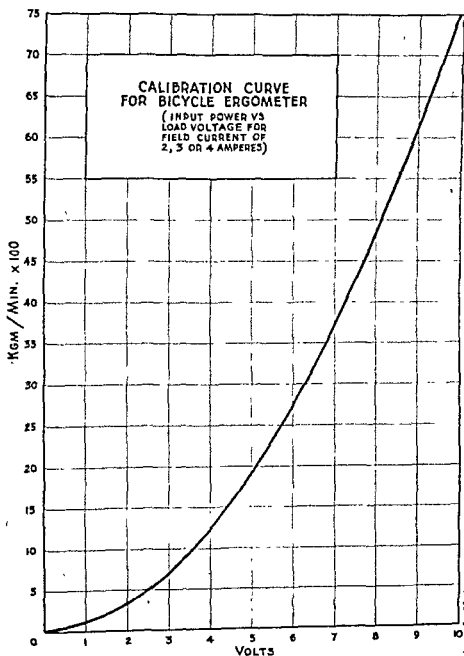


Fig. 3.—Curve for the conversion of recorded terminal voltage into work rate in kilogram meters per minute.

*Drawing the Calibration Curve.*—Since the power input to the ergometer is equal to the sum of the mechanical losses, armature losses, and load resistor losses, these values are summed up and this quantity is the total power input to the generator system for the specified terminal voltage. By computing a number of these quantities and plotting them against their respective voltages, a calibration curve may be drawn (see Fig. 3).

Power values computed in this manner will, of course, be in watts and if the curve is to be in terms of kilogram meters per minute, the power values must be multiplied by the conversion factor of 6.15. The figures presented in Table I are based upon the calibration curve shown in Fig. 3. They are used to convert terminal voltage to work rate or power in kilogram meters per minute for field currents between 2 and 4 amperes.

TABLE I

WORK RATE EQUIVALENTS IN KILOGRAM METERS PER MINUTE FOR GENERATED TERMINAL VOLTAGE (2 TO 4 AMPERES FIELD CURRENT)

VOLTS	KG.M./MIN.	VOLTS.	KG.M./MIN.
2.0	340	6.0	2,760
2.2	400	6.2	2,940
2.4	470	6.4	3,130
2.6	550	6.6	3,330
2.8	630	6.8	3,530
3.0	720	7.0	3,740
3.2	810	7.2	3,950
3.4	910	7.4	4,170
3.6	1,020	7.6	4,400
3.8	1,130	7.8	4,630
4.0	1,250	8.0	4,870
4.2	1,370	8.2	5,120
4.4	1,500	8.4	5,370
4.6	1,640	8.6	5,630
4.8	1,780	8.8	5,890
5.0	1,930	9.0	6,160
5.2	2,080	9.2	6,440
5.4	2,240	9.4	6,720
5.6	2,410	9.6	7,000
5.8	2,580	9.8	7,290

#### TECHNIQUE FOR MEASURING WORK RATE (POWER) AND WORK OUTPUT

The bicycle ergometer described in this paper is adaptable to many uses in research relating to the working efficiency of the human body. Most investigations in this field of experimentation have been concerned with the physiologic effects of working at a constant rate for prolonged periods. The automatic recording feature of the apparatus makes it possible to determine constancy of work rate and total amount of work performed. Other studies have attempted to approximate the optimum rate and load for working under various conditions. The ergometer provides an accurate method of measuring the work rate, work load, and total work output.

Recently, studies in the field of physical fitness have employed the bicycle ergometer to measure work rate and total output during short periods of working at maximum speeds. Since dynamic physical fitness may be defined as the capacity to do work, the ergometer provides reliable and valid criteria of this type of physical fitness. The findings of these studies are as yet inconclusive, but they seem to indicate that in some cases maximum total work output for a period of about two minutes is in itself a valid criterion of dynamic physical fitness. Other findings indicate that work output relative to body size and strength is one of the most valid measures of dynamic physical fitness.

The chief purpose of the bicycle ergometer in all these investigations has been to obtain a reliable estimate of the work rate and work output. The technique for obtaining these measures from continuous voltage recordings as obtained from the bicycle ergometer is described in the next paragraphs.

*Determining Work Rate (Power).*—A typical voltage curve obtained by pedaling the bicycle ergometer at top speed for two minutes is reproduced in Fig. 4. The tracing is a continuous recording of the generated voltage. The small fluctuations coincide with vibrations in the apparatus and the uneven application of force to the pedals and may be disregarded. The larger fluctuations represent changes in work rate due to fatigue and alterations in effort on the part of the subject.

From the record shown in Fig. 4 it is possible to obtain the generated voltage at any instant during the two-minute working period represented by this tracing. By referring to Table I, this instantaneous voltage can be converted to work rate or power in kilogram meters per minute. This measure, in itself, is interesting and of value in certain types of investigations, but its chief value lies in its use for determining work output for definite working periods.

*Determining Work Output.*—The record shown in Fig. 4 also represents the fluctuations in work rate for a working period of two minutes. To obtain the work output for all or any part of the two-minute period, it is necessary to redraw this curve so that the area under all parts of the curve will represent the work done (see Fig. 5). This is essential because the generated voltage is not a linear function of the power input to the machine.

To accomplish this, the following steps must be carried out: First, obtain voltage readings at regularly spaced moments throughout the working period and by referring to Table I convert these voltage readings into work rates in kilogram meters per minute. Next, replot the curve with work rate on the y-axis and time on the x-axis. The result is a work curve, and the area under the curve, being the product of work rate and time, is proportional to the work output. Therefore, the final step is to measure the area under all or any part of the curve with the aid of a planimeter and to multiply this area by the appropriate conversion factor. The resultant will be the work output for the period of time represented by the section of the work curve marked off.

Since the conversion factor mentioned in the previous paragraph is dependent upon the scales used in plotting the work curve, the following illustration is presented to clarify the last-mentioned step: Let us assume that our replot is made upon cross-section paper which is ruled off in millimeters and that 1 cm. on the vertical axis represents 100 kg. meters per minute work rate. Now if the horizontal axis is laid off so that 10 cm. represents one minute of elapsed time, 10 sq. cm. of area under the curve will be equal to 100 kg. meters of work and 1 sq. cm. will be equal to 10 kg. meters of work. The value 10 then becomes the conversion factor when the planimeter is adjusted to measure area in square centimeters.

This method for measuring work output is recommended when a planimeter is available. However, this instrument is not always accessible so that the following substitute method may prove more feasible: Proceed by marking off five-second intervals on the voltage tracing. Then obtain an average voltage

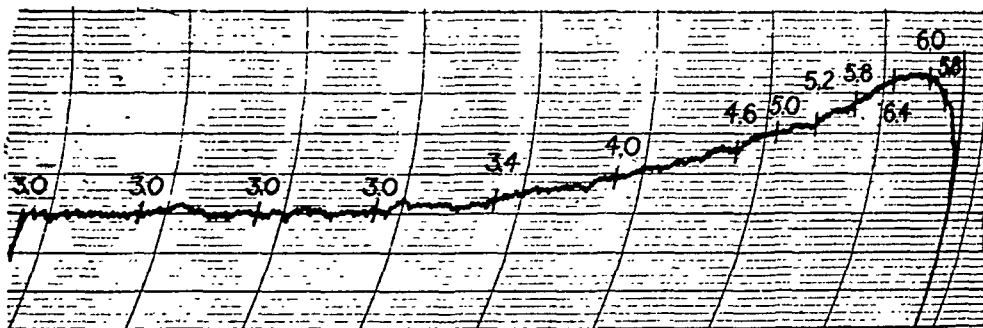


Fig. 4.—A typical voltmeter recording showing the continuous generated voltage during a two-minute working period on the bicycle ergometer.

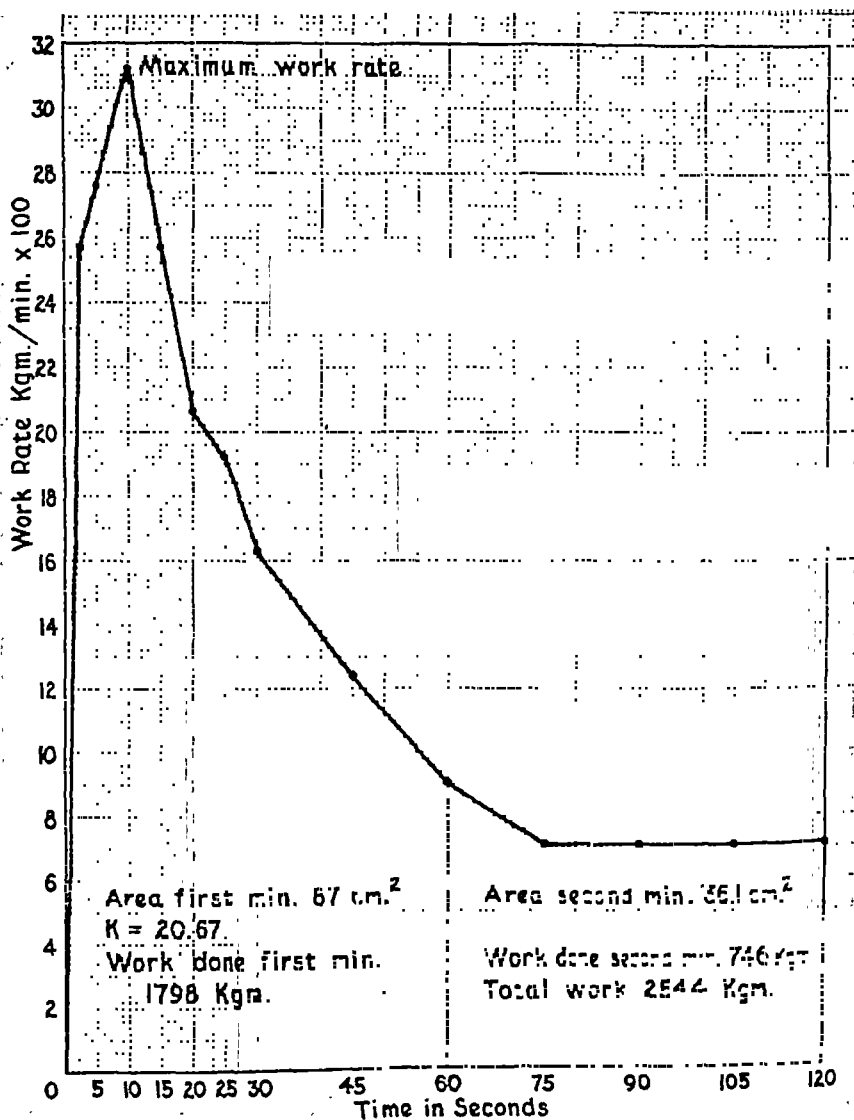


Fig. 5.—The plotted work curve for the voltmeter recording shown in Fig. 4.

reading for each interval, and by referring to Table I convert these readings to work rate values. The mean of these values will then represent the average work rate during the working period. The product of this average work rate and the elapsed time in minutes is a reliable estimate of the work performed. While this approach is not as accurate as the first-mentioned method, especially when the voltage curve fluctuates markedly, the results do not, as a rule, deviate by more than 2 per cent from planimeter measurements. This is well within the expected error for similar measurement techniques.

*Using the Ergometer for the Measurement of Physical Efficiency.*—The bicycle ergometer lends itself well as a method of work in determining physical efficiency by the oxygen consumption method. The most satisfactory work rate has been found to be 1,500 kg meters per minute for one minute. This means a pedal rate of 60 revolutions per minute controlled by having the subject work in rhythm with a metronome set to produce 120 clicks per minute. The limiting factor in the determination of physical efficiency by this method is the spirometer from which the oxygen is breathed. To provide enough oxygen for a greater work rate than 1,500 kg-meters per minute for one minute necessitates enlarging the outlet of the spirometer. This in turn results in a large dead air space and causes rebreathing of expired air. The respiration of the subject is so affected by this rebreathing that the experiment cannot be carried to completion successfully.

The physical efficiency of the average individual, as measured by the oxygen consumption method using the bicycle ergometer as the method of work, has been found to vary between 20 to 25 per cent.

#### SUMMARY

The construction, calibration, and use of an alternating current electrodynamic brake bicycle ergometer are described. In addition, techniques for measuring work output and maximum work rate are discussed. Instructions for the care and standardization of the machine are included.

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## A DEVICE FOR RECORDING THE RESPIRATION OF ANESTHETIZED LABORATORY ANIMALS

LIEUTENANT CARL C. PFEIFFER, MC-V(S), USNR., BETHESDA, MD., AND  
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THE usual pneumograph for recording the respiration of laboratory animals requires a sensitive and airtight tambour and has the disadvantage that it may be dislodged from a fixed position on the chest. This device may also fail to record diaphragmatic breathing if, due to increased anesthesia, the intercostal muscles become paralyzed. The pleural cannula\* has the disadvantage of producing some trauma to the pleura or lung, and if the tambour is not airtight, unsuspected pneumothorax may result. Other respiratory recording devices tend to be complicated and cumbersome.

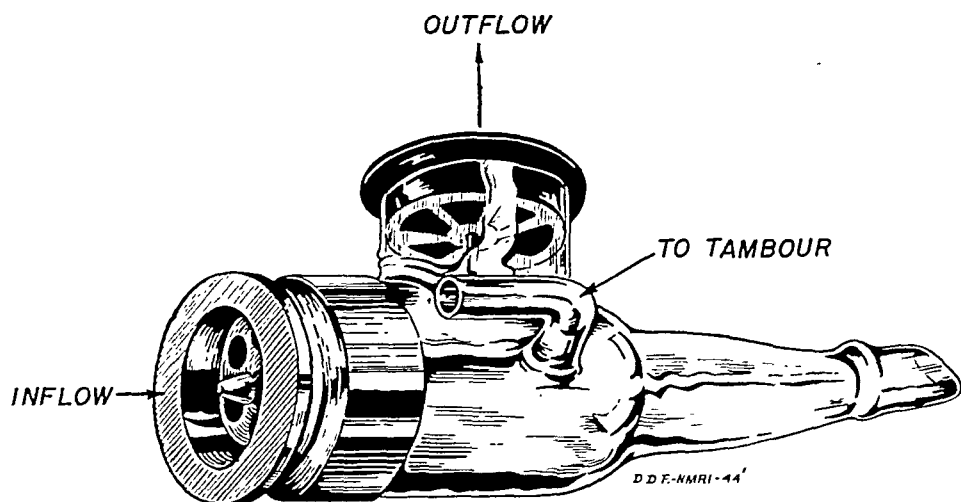


Fig. 1.—Modified tracheal cannula (two-thirds actual size).

Several years ago a suitable tracheal cannula modified for recording respiration was constructed using a two-way system of wedge-shaped flutter valves with a side arm connecting to a tambour. When used in large dogs, however, the valves did not allow for adequate respiratory exchange. With the advent of improved flutter valves, such as are now used in aviators' oxygen masks (M-1 and A-10 valves),† an accurate and efficient device can be easily constructed.

\*Mendenhall, W. H.: Science 86: 129, 1937.

†Acushnet Processing Co., Bridgeport, Conn.

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Due to the present shortage of metallic materials, the model pictured in Fig. 1 is made almost entirely of glass. A more durable recorder could be made of brass or plastic. The recorder produces a constant record of respiration with

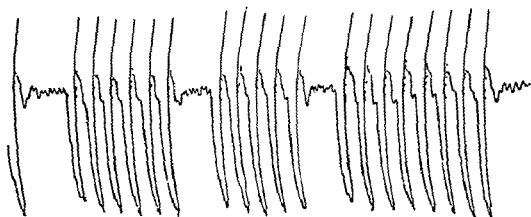


Fig. 2.—Typical recording of respiration and pulmonary pulse (actual size).

any of the usual laboratory tambours (Fig. 2). Inspiration is recorded by the downstroke and expiration by the upstroke. Since both valves are closed between respiratory cycles, the pulmonary pulse usually is recorded. This may at times be advantageous. By means of a slip joint on the inflow flutter valve, the composition of the atmosphere supplied to the animal may be varied at will.

## PHYSIOLOGICAL SALINE WITHOUT WEIGHINGS

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THIS time-saving and labor-saving method of preparation, well adapted to field use, was developed to expedite filling of the hospital's large requirements for saline for lavages, rinsing of special surgical equipment, etc. Subsequently the saline so prepared was found entirely suitable for all laboratory and clinical uses as well, and the method has been employed with satisfaction for the past twelve years.

The chemical principle made use of, peculiar to sodium chloride, is its slight solubility change with change of temperature. The solubility plotted against temperature yields a straight line,<sup>1</sup> and over the extreme temperature range from 0 to 100° C. rises only from 35.7 to 39.8 Gm. per 100 Gm. water.<sup>2</sup> For a 10° temperature interval (ordinary room temperature range) the over-all change in solubility is approximately 1 per cent of the total, and the ultimate dilution of the finished saline is such that the actual weight of salt varies only by 0.01 Gm. per 100 c.c. The several concentrations which have been recommended by various authorities as "normal saline" (from 0.85 to 0.90 per cent) are much farther apart than this, differing in fact by more than 5 per cent, or 0.05 Gm. per 100 c.c.

A saturated solution of sodium chloride in distilled water, above a thick layer of excess salt, is maintained in a large stock bottle with salt or water added as needed. The saturated brine is filtered off from time to time into another stock bottle and the saline is prepared from this filtrate by dilution.

Since 100 Gm. of water containing over 35 Gm. of sodium chloride no longer occupied 100 c.c., it was necessary to work out the new weight-volume relationships and determine by accurate gravimetric methods the salt content per unit of volume. The calculations were made for a physiological saline of 0.90 per cent concentration.

Dilution of 2.60 c.c. of filtered saturated brine to 100 c.c. with distilled water produces a saline containing exactly 0.90 Gm. sodium chloride per 100 c.c. In titration with silver nitrate, using chromate as indicator, 2.00 c.c. of this saline are equivalent to 10.91 c.c. of M/35.46 silver nitrate.

Over the temperature range from 0 to 47° C., or from 32 to 117° F., the total change in concentration of the finished saline still remains no greater than that between 0.85 and 0.90 per cent saline. For nearly absolute constancy of concentration, the volume of brine used can be increased by 1 per cent for each 10° interval below 18° C. and decreased by 1 per cent for each 10° interval above 28° C. For all practical purposes, such correction is unnecessary.

Sterilization of saturated brine by autoclaving or boiling does not affect the concentration, as loss of water leaves the solution still saturated. On the other hand, the application of heat in preparing the brine from the dry salt is

From the Wm. H. Maybury Sanatorium (Detroit Municipal Tuberculosis Sanatorium).  
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of no particular benefit due to the special solubility-temperature relationship described. Agitation, to get the heavy layers off the bottom, is the main necessity in obtaining saturation; a shaking machine may be used, but an occasional roll or shake of the stock bottle by hand is effective.

A further advantage in the use of saturated sodium chloride brine is the ease of making a saline from some other solution (nutrient or antiseptic) with only negligible dilution of the other component; sterile filtered brine, in the small volume required, again 2.60 c.c. per 100 c.c., can be added quickly with sterile technique.

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## MEDICAL ILLUSTRATION

### LIGHT SOURCE FOR FLUORESCENT MICROSCOPY

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CHICAGO, ILL.

A STRONG source of violet light is desirable in using the fluorescent method of detecting tubercle bacilli. Most recent work seems to have been done with the 100-watt AH4 mercury lamp. However, Kloeck and Sweany<sup>1</sup> have obtained much better results with the 250-watt CH5 lamp. Both of these lamps are made with a quartz light capsule sealed in a clear tubular bulb. The CH5 lamp is considerably larger than the AH4.

Another lamp which has several useful features is the 100-watt CH4 lamp.\* In this lamp the quartz capsule is sealed in a bulb with an aluminum reflector, similar to a sealed beam headlight. The construction is such that a strong spot of light is obtained. In this arrangement the lamp supplies its own housing and efficient optical system. It is necessary only to support the lamp from a laboratory stand and to supply current from the proper transformer.

Some comparative tests have been made which should be helpful in using these lamps. A metal plate with a 0.5 mm. hole drilled through it was fitted into the mechanical stage of the microscope. A selenium photocell was fastened above this hole and connected to a microammeter. With this apparatus, the light intensity over a small area of the field could be determined. All readings were made with a Corning No. 5113 violet filter fastened under the condenser.

The distance between the CH4 lamp and the microscope mirror was varied and the photocell readings noted. Over a range of from 10 to 25 cm. the readings were almost constant.

When using the CH4 lamp it will be noted that the pattern of the field as observed in the top of the condenser consists of a black center surrounded by a broad ring of light. The black center corresponds to the opaque end of the light capsule mounted in the bulb. The ring of light is not uniform in intensity, but an intense area can be brought into the field by adjustment of the microscope mirror.

Similar tests were made with the AH4 lamp. By allowing this lamp to burn freely in the air it was found the intensity dropped rapidly as the lamp was moved away from the microscope. At 3 cm. from the microscope mirror the intensity was nearly equal to the CH4 lamp, whereas at 10 cm. the intensity was 25 per cent of this value.

Dr. H. C. Sweany, of the Chicago Municipal Tuberculosis Sanitarium, kindly made their CH5 lamp available for measurement. This lamp was

From the Department of Medicine, University of Chicago Clinics.

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\*Listed by General Electric Co. and Westinghouse Electric and Manufacturing Co.

mounted in a housing with a mirror in the rear and a condensing lens in front. The photocell gave readings nearly equal to that of the CH<sub>4</sub> lamp.

The use of an aluminized microscope mirror has been suggested to increase the illumination. A nonfluorescent liquid, such as water, placed between the condenser and slide also increases the illumination. However, the photocell indicated that neither of these methods would cause a 10 per cent increase. Visual tests indicated that the increased illumination was not especially noticeable.

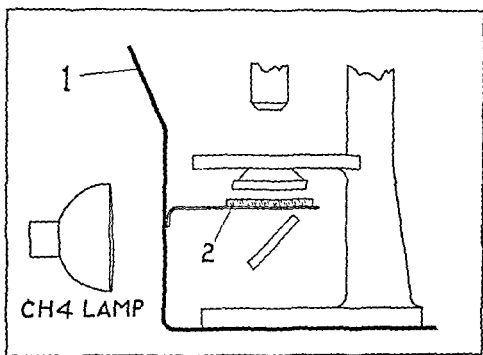


Fig. 1.—Simple apparatus for fluorescent microscopy.

The following notes are the result of experience obtained in the University of Chicago Clinics from tests of the fluorescent method for detecting tubercle bacilli.

An ordinary microscope can be readily converted into a fluorescent microscope, as shown in Fig. 1. The CH<sub>4</sub> lamp is sufficiently intense for binocular vision. A simple light shield, 1, is required. Filter 2, made of Corning No. 5113 violet glass, is mounted under the condenser. A filter of Corning No. 3486 yellow is supported under the eyepiece. Both filters are of "standard" thickness.

The stained slides are readily scanned with the low-power objective, and final identification of the tubercle bacillus is made with the oil immersion lens.

To avoid the use of oil, a 45 × N. A. .95 objective was tested. The resolving power of this lens was inferior to the oil lens but might be considered satisfactory. It is important that such a lens not be corrected for cover slip thickness.<sup>2</sup>

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## A NOTE ON INFRARED PHOTOGRAPHY OF CUTANEOUS ARTERIAL "SPIDERS" AND HEREDITARY HEMORRHAGIC TELANGIECTASES

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THE infrared technique has been used widely to give photographic accentuation to dark red or bluish structures on or near the surface of human skin. Collateral venous channels not visible to the unaided eye have been demonstrated over the abdomen in cirrhotic patients with ascites; and the large networks of veins over the breasts and anterior thoracic wall of pregnant women have been depicted in infrared photographs, whereas they do not appear in ordinary black-and-white pictures or appear but faintly.

During a study of several hundred examples of the cutaneous arterial "spiders",<sup>1, 2</sup> it occurred to me that the opposite effect might be obtained, namely, infrared photographs might eliminate bright red structures which were recorded in a satisfactory manner by means of ordinary photography. Such proved to be the case. The arterial "spider" of the skin has a bright red color which depends on the abundant supply of arterial blood brought unusually near the surface of the skin in thin-walled vessels. In Fig. 1 black-and-white and infrared photographs of the same area of the forearm and antecubital region of a patient with advanced cirrhosis are compared. A number of "spiders," some large and some small, stand out in clear detail in the black-and-white photograph. They do not appear, except for a faint ghost marking the site of the body of the spider, in the infrared photograph. This photographic comparison has been carried out several times with precisely the same results.

Although the arterial nature of the vascular "spider" of the skin has been known for more than fifty years, and pulsation can be detected in the larger ones, this technique offers a diagnostic aid in establishing the nature of small, nonpulsatile, or atypical lesions.

In Fig. 2, the lesions of hereditary hemorrhagic telangiectasia (Osler's disease) are compared by the same technique. It is readily apparent that the same phenomenon occurs when this type of lesion is photographed by the infrared method as when the arterial "spider" is photographed in black and white, namely, it does not appear in the picture. This is of interest since the color of the smaller punctate lesion of Osler's disease is usually more of a red-blue or violaceous color than the arterial "spider," which is regularly a bright scarlet color, from the oxyhemoglobin. In both cases this method gives additional evidence that the vessels chiefly affected are on the arterial side of the capillaries and carry arterial blood. It is possible to bring out the gradations in color and the contrasts between the "spider" and the hereditary telangiectases by the use of Kodachrome and other color films, but the exact reproduction of

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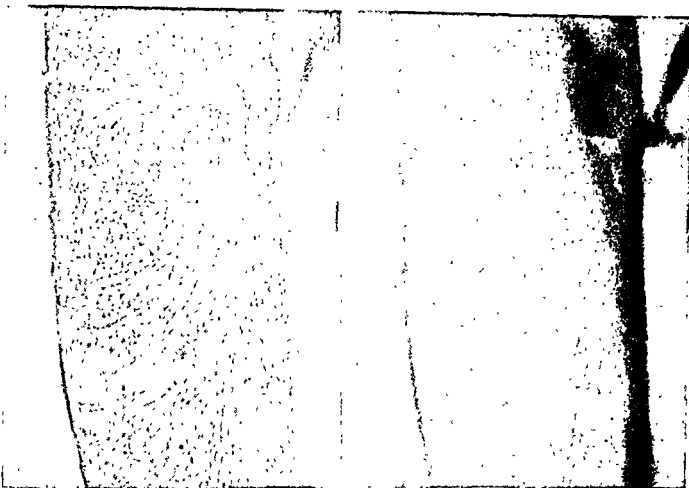


Fig. 1.—A comparison of black-and-white (left) and infrared (right) photographs of the forearm of a patient with cirrhosis showing the failure of the lesion to appear in the infrared photograph.



Fig. 2.—A comparison of black-and-white (left) and infrared (right) photographs of a person with Osler's disease—*hereditary hemorrhagic telangiectasia*. Lesions on the tongue and cheek fail to appear except as ghost marks in the infrared photographs.



colors is still difficult technically. For that reason, comparison of black-and-white and infrared photographs offers a simple method of establishing the arterial or venous nature of vascular lesions of the skin and mucous membranes.

#### CONCLUSION

The fact that the arterial "spider" of the skin does not appear in infrared photographs suggests a use in segregating it from venous telangiectases, though they will not serve to differentiate it from the telangiectasis of Osler's disease.

I am indebted to Mr. Joseph B. Homan, Assistant Professor of Medical Photography, for taking the pictures.

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## BOOK REVIEWS

**A Textbook of Pathology.** By *Robert Allan Moore*, Edward Mallinckrodt Professor of Pathology, Washington University School of Medicine. W. B. Saunders, Philadelphia, Pa. Price \$10.00. Cloth with 1,338 pages with 513 illustrations, 34 in color

This textbook of pathology, the latest to appear in the American literature, is well printed and illustrated and should take its place as one of our foremost texts. The author has made it more attractive than some textbooks by including a considerable number of colored plates, as well as radiographs, photographs of patients, and reproductions of frontispieces from classical monographs, thus reflecting his wide experience both in the teaching and study of pathology in this country and abroad. As a result the book lacks some of the provincialities too frequently manifested in certain textbooks. The arrangement of the book includes both general and special pathology, and the emphasis throughout is predominantly on etiology. In association with the discussion of the various pathologic entities there is usually a clinicopathologic correlation. The author has also assembled at the end of each chapter a selected list of references, most of which are relatively recent. Students using these, therefore, can do so with the assurance that they are studying pathology as a developing science. Throughout the book the author demonstrates his purpose of keeping abreast of the current literature and of describing pathologic changes not only in their relationships to morphology, but also to biochemistry, physiology, bacteriology, and clinical medicine. As with every textbook, the discussions have been necessarily restricted, but the author has shown good discrimination in his selection of material. The book may be recommended as a reliable and well-planned textbook with the particular merit of emphasizing dynamic aspects of pathology.

P. R. C.

**Familial Susceptibility to Tuberculosis: Its Importance as a Public Health Problem.** By *Ruth Rice Puffer*, Dr.P.H., Tennessee Department of Public Health. Harvard University Press, Cambridge, Mass., 1944. Price \$2.00. Cloth with 106 pages.

Isolation of the diseased patient—the prevention of contact—has been the principal method used in public health programs for the control of tuberculosis. But, as Dr. Puffer states in her introduction: "Although isolation reduces the opportunities of exposure of the population, this measure has not been sufficient to prevent the majority of the population in many sections of the country from becoming infected before reaching adult life. Since we cannot prevent all contact with tubercle bacilli, we need to go deeper into the problem to understand the conditions under which infection results in disease." She attempts

to do this in part by analyzing the importance of familial susceptibility to tuberculosis and suggesting how this information may be used in an attempt to make the tuberculosis control program more effective.

The literature is reviewed and new data from the Williamson County (Tennessee) Tuberculosis Study are presented. Her analysis of data for siblings, consorts, parents, and children of tuberculous patients suggests that hereditary or familial susceptibility is a determinant in the development of the disease. The families of consorts were studied as one of the control groups. Dr. Puffer emphasizes that the development of tuberculosis must be studied as a manifestation of a patient's total life experience. If the attack rate is studied in children of tuberculous patients, for instance, it is not enough to terminate the study when the child leaves his parents' home to establish one of his own. She points out that "while children of a sputum-positive parent developed tuberculosis very frequently in childhood, children of a parent with tuberculosis other than sputum-positive developed the disease frequently in adult life. By 50 years of age approximately the same proportion of both groups of children had developed tuberculosis."

The data available for this study indicate that the incidence of tuberculosis is as great among susceptible families as it was before the turn of the century. Dr. Puffer suggests that since the tuberculosis death rates of female children of tuberculous parents are particularly high, these families tend to die out. The decline in tuberculous death rates, therefore, may be due in large part to the reduction of susceptible families in the population.

In most existing public health programs, only the household associates of a patient who develops tuberculosis are examined and followed. The studies summarized in this monograph indicate that these programs should be augmented so that "siblings, parents, and children of persons known to have tuberculosis or to have had the disease in the past be examined and followed irrespective of their present household."

Dr. Puffer presents her argument in a simple and convincing manner. Her book should prove as stimulating and helpful to the clinician as to the public health physician. It is a worthy companion to the other Harvard University monographs in medicine and public health.

C. V. M.

**The Avitaminoses: The Chemical, Clinical and Pathological Aspects of the Vitamin Deficiency Diseases.** By *Walter H. Eddy*, Ph.D., Emeritus Professor of Physiological Chemistry, Teachers College, Columbia University; and *Gilbert Dalldorf*, M.D., Pathologist of the Grasslands and Northern Westchester Hospitals, Westchester County, N. Y. Third Edition. The Williams & Wilkins Company, Baltimore, 1944. Price \$4.50. Cloth with 438 pages.

**Medical Uses of Soap.** Edited by *Morris Fishbein*, M.D., J. B. Lippincott, Philadelphia. Price \$3.00. Cloth with 182 pages, 41 illustrations.

# THE RELATION OF PERIARTERITIS NODOSA TO BRONCHIAL ASTHMA AND OTHER FORMS OF HUMAN HYPERSENSITIVENESS\*

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THE first case of periarthritis nodosa was described by Kussmaul and Maier<sup>1</sup> in 1866. This condition was recognized so infrequently that fifty-seven years later (1923) a review of the literature revealed some seventy references to the disease.<sup>2</sup> By 1935, 200 cases were assembled,<sup>3</sup> 1936, 215 cases,<sup>4</sup> 1939, 245 cases,<sup>5</sup> and through 1940, 350 cases.<sup>6</sup> In view of the earlier apparent rarity of reported instances, it is remarkable that some 200 authenticated cases were described from early 1940 through 1943. As a matter of fact, the disease is coming to be recognized so readily that single case reports now occur largely in state journals and other less prominent periodicals, whereas various authors have recently reported series of cases as many as fourteen in one man's experience.<sup>7</sup>

There have been many theories concerning etiology. That of hypersensitiveness was suggested by Gruber,<sup>8</sup> who in 1925 induced typical arterial lesions experimentally in sensitized animals. Klinge,<sup>9</sup> Masugi and Isabasi,<sup>10</sup> Rich and Gregory,<sup>11</sup> Selye and Pentz,<sup>12</sup> and others likewise succeeded in provoking periarthritis in various species of animals who had been first sensitized and then reinjected. Numerous antigens such as bacteria, horse serum, and tissue extracts were used.

In man, the lesions of periarthritis nodosa have been found at autopsy or on biopsy from patients whose presenting symptoms were those of various forms of allergy. Although this fact had been noted many times, the role of human hypersensitiveness has been treated broadly and no apparent attempt has been recorded to relate periarthritis to the several specific types of hypersensitiveness. These include atopy, atopic-like disorders (intrinsic allergy), drug allergy, serum sickness, bacterial allergy, and contact dermatitis. Although there is dispute as to whether or not some of these are expressions of one underlying process, it matters little to the purpose of this presentation.

*Atopy and Atopic-like Disorders (Intrinsic Allergy).*—Atopic-like disorders, which include bronchial asthma, chronic urticaria, and chronic vasomotor rhinitis, may be clinically identical to those of atopy. The particular difference is that atopy is mediated by a definite immunologic process with distinctive antibodies and antigens such as foods and pollens that are extrinsic to the body. In nonatopic or atopic-like disorders there is no identifiable immunologic

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mechanism, and the underlying process presumably originates intrinsically. This difference is stressed for the reason that most cases of asthma identified with periarteritis nodosa belong to the latter group.

An analysis by the authors of 300 consecutive cases of periarteritis nodosa, beginning with the first case of bronchial asthma reported some thirty years ago, revealed many instances of associated atopy and atopic-like disorders. Particular attention was paid to bronchial asthma of which there were fifty-four cases, an incidence of 18 per cent. This is a considerably higher incidence than that found by Rackemann and Greene<sup>5</sup> who reviewed the literature up to 1939. Of 229 references, they discovered nineteen cases of asthma to which Rackemann added eight more, giving a total incidence of 12 per cent. It is of interest that during the four years following their report we found twenty-seven more cases, or exactly the same number that had appeared during the period of sixty-eight years embraced by Rackemann and Greene. Furthermore, we found many typical case reports wherein asthma and periarteritis were associated but not confirmed by autopsy or biopsy. Were these included, the incidence of bronchial asthma with periarteritis nodosa would exceed 20 per cent of the reported cases. Many instances of urticaria, vasomotor rhinitis, and other expressions of atopy were encountered. If they were included with asthma, the incidence of atopy and atopic-like disorders accompanying periarteritis nodosa would be well over 25 per cent.

Analysis of case reports reveals that in almost every instance asthma antedated periarteritis; in a few cases, by many years, but in the great majority, by but a few years, and sometimes by only a month or two. In three cases symptoms of asthma followed the onset of periarteritis, but whether or not these were the first attacks of asthma was not stated. In Table I is recorded the ages of patients at the time of onset of periarteritis nodosa. Inasmuch as asthma was then of comparatively short duration in most instances, the age when bronchial asthma first appeared is also roughly recorded in Table I since only decades are stated. It is of interest that the onset occurred before the age of 21 in but 11 per cent, whereas some 70 per cent of all cases of asthma begin in the first two decades.

TABLE I

AGE (IN DECADES) OF PATIENTS WITH PERIARTERITIS NODOSA WITH AND WITHOUT BRONCHIAL ASTHMA

DECADE	ASTHMATIC GROUP		NONASTHMATIC GROUP	
	NUMBER OF CASES*	PER CENT OF CASES	NUMBER OF CASES†	PER CENT OF CASES
First	2	4.3	26	11.6
Second	3	6.4	24	10.7
Third	10	21.3	40	17.7
Fourth	16	34.0	55	24.4
Fifth	6	12.8	38	16.9
Sixth	8	17.0	23	10.2
Seventh	2	4.3	16	7.1
Eighth	0		3	1.3
Average age	35.4 years		35.7 years	

\*In seven cases age not stated.

†In twenty-one cases age not stated.

The asthmatic paroxysms were usually severe and rarely were extrinsic allergens as foods and pollens identified as definite etiologic agents. These two facts, coupled with the later age of onset and the hypereosinophilia, identify most of the reported cases as nonatopic or intrinsic asthma.

Perhaps the most remarkable feature of the cases of asthma associated with periarteritis nodosa was the marked hypereosinophilia in the blood (of most). Values exceeding 80 per cent of the total white blood count have been recorded. Of the fifty-four patients with asthma, forty-four had a blood eosinophilia of over 11 per cent, and the majority were far above this value.

TABLE II

TOTAL LEUCOCYTE COUNT AND PERCENTAGE OF EOSINOPHILES IN CASES OF PERIARTERITIS NODOSA AND BRONCHIAL ASTHMA

CASE	AUTHOR	MAXIMUM WHITE BLOOD COUNT	MAXIMUM PERCENTAGE OF EOSINOPHILES
1	Lamb (1914) <sup>11</sup>	20,900	51
2	Laux (1925) <sup>14</sup>	12,600	12
3	Curtis and Coffey (1934) <sup>15</sup>	19,900	37
4	Grill (1934) <sup>16</sup>	20,000	30
5	Taylor and Farley (1934) <sup>17</sup>	25,700	72
6	Middleton and McCarter (1935) <sup>3</sup>	33,950	72
7	Bahrman (1935) <sup>18</sup>	15,000	60
8	Cohen, Kline, and Young (1936) <sup>19</sup>		30
9	Motley (1936) <sup>4</sup>	32,000	67
10	Leishman (1937) <sup>20</sup>	11,000	55
11	Baehr and Klemperer (1937) <sup>21</sup>	*	*
12	Strong (1938) <sup>22</sup>	32,000	79
13	Fitz, Parks, and Branch (1939) <sup>23</sup>	15,000	50
14	Cabot Case Report (1939) <sup>24</sup>	18,000	43
15	Wier (1939) <sup>25</sup>	45,000	50
16	Rackemann and Greene (1939) <sup>5</sup>	18,000	43
17	Rackemann and Greene (1939) <sup>5</sup>	18,000	47
18	Rackemann and Greene (1939) <sup>5</sup>	26,000	50
19	Rackemann and Greene (1939) <sup>5</sup>	17,000	55
20	Rackemann and Greene (1939) <sup>5</sup>	26,000	70
21	Rackemann and Greene (1939) <sup>5</sup>	50,000	80
22	Rackemann and Greene (1939) <sup>5</sup>	16,000	37
23	Rackemann and Greene (1939) <sup>5</sup>	37,000	70
24	Dawson (1939) <sup>26</sup>		84
25	Trassoff and Scarf (1940) <sup>27</sup>	18,000	11
26	Lebowich and Hunt (1940) <sup>28</sup>	38,200	68
27	Cabot Case 26 (1940) <sup>29</sup>	18,900	47
28	Grant (1940) <sup>6</sup>	21,000	57
29	Coe, Reismann, and DeHoff (1941) <sup>30</sup>	14,600	40
30	Felsen (1941) <sup>31</sup>	32,000	80
31	Tissell (1941) <sup>32</sup>	20,200	49
32	Harkavy (1941) <sup>33</sup>	18,000	40
33	Harkavy (1941) <sup>33</sup>	19,000	39
34	Jones (1942) <sup>7</sup>		77
35	Jones (1942) <sup>7</sup>	14,500	23
36	Lund (1942) <sup>34</sup>		24
37	Baker (1942) <sup>35</sup>	24,550	70
38	Harkavy (1943) <sup>36</sup>	36,000	68
39	Harkavy (1943) <sup>36</sup>	20,000	39
40	Harkavy (1943) <sup>36</sup>	25,000	44
41	Harkavy (1943) <sup>36</sup>	40,000	50
42	Harkavy (1943) <sup>36</sup>	70,000	82
43	Harkavy (1943) <sup>36</sup>	*	*
44	Harkavy (1943) <sup>36</sup>	*	*
Average		25,860	53.5
Controls		19,100	2.5
(Cases without asthma)			

\*No blood counts recorded but hypereosinophilia stated to be present.

Of the ten patients with asthma without eosinophilia,<sup>3, 44-46</sup> no differential blood counts were recorded in seven, and increased eosinophiles may well have been present. In each of the remaining three instances only one count was reported with values of 6 per cent, 4 per cent, and 4 per cent of eosinophiles, respectively. Haining and Kimball<sup>37</sup> recommended repeated differential counts lest hypereosinophilia be missed. In a case of periarteritis nodosa and asthma reported by Lebowich and Hunt<sup>28</sup> there was no abnormal rise in eosinophiles until the disease was well advanced, when they appeared in large numbers. Of the 151 patients with periarteritis nodosa without asthma in which differential counts were recorded, but nine instances of hypereosinophilia appeared (Table III). This gives an incidence of 6 per cent in distinction to 94 per cent of the forty-seven cases with associated asthma.

TABLE III  
CASES OF PERIARTERITIS NODOSA AND HYPEREOSINOPHILIA WITHOUT ASTHMA

CASE	AUTHOR	TOTAL WHITE BLOOD COUNT	PER CENT OF EOSINOPHILES	REMARKS
1	Wohlwill (1923) <sup>39</sup>	"Normal"	16	Two other blood counts—normal values for eosinophiles; very inadequate history; examination of lungs not recorded
2	Haining and Kimball (1936) <sup>37</sup>	16,500	33	
3	Cabot Case Report (1936) <sup>38</sup>	35,000	35	Cough for years
4	Sandler (1938) <sup>40</sup>	21,000	55	Sniffles and nasal mucoid
5	Wegener (1939) <sup>41</sup>	2,200	20	discharge
6	Neumann (1940) <sup>42</sup>	—	68	Very brief report, examination of lungs not recorded; urticaria
7	Grant (1940) <sup>6</sup>	35,000	55	Nasal polyps and râles in lungs, but nature of these not mentioned
8	Payne (1941) <sup>43</sup>	17,050	17	
9	Jones (1942) <sup>7</sup>	18,000	28	

Hypereosinophilia of over 15 per cent in adults with uncomplicated bronchial asthma is unusual. Consequently, the association of bronchial asthma and a marked hypereosinophilia should lead to a strong suspicion of an underlying periarteritis nodosa.

*Drug Allergy.*—The production of periarteritis nodosa by drugs is, as yet, supported by little data. Rich and Gregory<sup>47</sup> observed a series of patients with pneumonia who received relatively large amounts of therapeutic serum together with sulfonamide drugs. At autopsy, typical lesions of periarteritis nodosa were found. The participation of the underlying infection was excluded by reproducing similar lesions in rabbits by injections of horse serum and sodium sulfadiazine. Although the experiment was successful with the administration of horse serum alone, Rich and Gregory encountered one patient who manifested typical arterial lesions after he had received sulfonamide without serum. In this case, tissue removed by biopsy at five months and one week before the sulfonamide reaction showed no periarteritis, whereas tissue study revealed typical widespread lesions nine days following the drug reaction. It is difficult to ascribe such a train of events to anything but drug allergy.

The question as to whether periarteritis caused by a sulfonamide drug is due to a hyperergic reaction or to a direct toxic effect will not be discussed

here, since it introduces the general problem of the pathogenesis of such drug reactions. However, the work of Hageman and Blake<sup>48</sup> and others<sup>49</sup> indicate that hypersensitiveness is the underlying mechanism.

*Serum Sickness.*—In 1937 Eason and Carpenter<sup>50</sup> found the lesions of periarteritis nodosa at autopsy of a patient who had had acute rheumatic fever treated with antistreptococcal serum. These authors believed that the vascular lesions were due probably to the rheumatic process rather than to the serum. In the same year Clark and Kaplan<sup>51</sup> reported two patients who developed serum sickness following the administration of large amounts of serum. They, too, presented periarteritis nodosa at post-mortem examination. Not only were there necrotising arteritis and periarteritis of the smaller coronary arteries, but proliferation of histiocytes in the mural and valvular endocardium. The following year Clark reported another case, one of acute poliomyelitis, in which a single dose of concentrated horse serum had been given both intraspinally and intramuscularly.

It was Rich and Gregory,<sup>11</sup> however, who demonstrated both clinically and experimentally that typical, extensive periarteritis nodosa may be produced by injections of foreign serum. They succeeded in causing the lesion to appear in rabbits following a single large injection of horse serum. In such instances, free antigen remained in the blood stream after specific antibodies had developed.

Rich and Gregory's observations are surprising in that after forty years of serum therapy, they were the first to identify, definitely, periarteritis as a consequence of serum treatment. There doubtless have been thousands of autopsies on patients who had received therapeutic sera and died soon thereafter, and yet the lesions of periarteritis nodosa, which are too obvious to escape detection, were described first in 1937. Rich and Gregory's series of cases is, therefore, remarkable. Two possibilities concerning the production of periarteritis in these cases present themselves. One involves the question as to whether sulfonamide drugs enhanced the action of serum on vascular tissues. Rich and Gregory commented on this possibility but believed it improbable because they were able to produce typical lesions in rabbits with serum alone just as readily as when the drug was used in addition. The second possibility concerns the large amounts of serum that were given both clinically and in experimental animals, a fact which the authors also recognized, and they pointed out that Clark and Kaplan's two reported patients likewise were given serum in large quantities. Although both precipitins and "serum sickness antibody" are usually present in the blood, attempts to correlate immune reactions with the extent of periarteritis present were not made in any of the cases studied.

*Bacterial Allergy.*—Bacterial allergy is exemplified by such lesions as the tuberculin skin reaction, rheumatic fever, and the "id" of various fungus infections. The term infectious asthma is often used on the assumption that asthma in the presence of respiratory tract infection is, likewise, due to bacterial hypersensitiveness, although proof of this theory is lacking. Certainly the mechanism involved in such cases of asthma is obscure and is different from those due to atopy wherein an antigen-antibody reaction is demonstrable. Of importance insofar as periarteritis nodosa is concerned is the consideration as to whether the lesions of acute rheumatic fever are a hyperergic response



to the product of streptococcal growth or to other antigens. Swift and his associates<sup>52</sup> assembled evidence to support this theory and it has gained wide acceptance.

Rheumatic fever and periarteritis are linked together by three facts. One concerns the frequency with which both clinical symptoms of rheumatic fever and typical Aschoff bodies have been found with periarteritis nodosa. Klinge,<sup>9</sup> Friedberg and Gross,<sup>53</sup> Spiegel,<sup>44</sup> and others have noted this association. The second fact is that in instances of severe rheumatic fever with involvement of the blood vessels, rheumatic arteritis is at times indistinguishable from periarteritis nodosa. In each there may be fibrinoid degeneration, palisading, and necrosis of the intima and signs of intense perivascular inflammation. One possible distinction has been commented upon:<sup>54</sup> namely, that with nonbacterial allergens, eosinophiles are numerous, whereas in rheumatic arteritis they are not, but the necrosing tissue is eosinophilic. This point needs elaboration although Rich found eosinophiles in his experimental animals. Finally, lesions of both rheumatic fever and periarteritis have been produced experimentally by injections of foreign serum into rabbits. Klinge<sup>9</sup> observed arteritis which he believed resembled that of rheumatic fever as well as a vascular lesion simulating periarteritis after repeated injections of horse serum. Metz<sup>55</sup> induced periarteritis nodosa by injection of cattle serum and recognized rheumatic lesions in some of his animals. Rich recently discovered typical Aschoff bodies in rabbits injected with a large amount of horse serum.

These experiments wherein the typical Aschoff bodies of rheumatic fever have been produced through the process of sensitization with nonbacterial antigens have important implications. They strongly support the theory that the lesions of rheumatic fever express a hyperergic reaction and they may reveal the mechanism, as yet unknown, of bacterial hypersensitiveness.

Although, clinically, periarteritis nodosa itself presents signs of infection such as fever, acute arthritis, and usually a marked leucocytosis, no specific infectious agent has been identified with it.

*Contact Dermatitis.*—We encountered no case of contact dermatitis with periarteritis nodosa. This is not surprising since the eczematous lesion of contact dermatitis is confined largely to the skin epithelium where the only blood vessels present are capillaries.

#### DISCUSSION

In previous reports dealing with hypersensitiveness and periarteritis nodosa, no consideration has been given to the identification of the particular types of allergy involved. Rich implied certain forms by stating that the arterial lesions resembled "hives of the blood vessels." Such a lesion (whealing) occurs in atopy and atopic-like disorders, serum sickness, and, occasionally, in drug reactions. It is by no means typical of bacterial allergy.

Previous to this investigation no apparent factor common to all forms of human hypersensitiveness had been apparent. It is of interest that periarteritis nodosa appears to be the only evident manifestation which links all types of human hypersensitiveness together with the exception of contact dermatitis. This fact points again to the role of the blood vessels in hypersensitive states.

Roessle<sup>56</sup> demonstrated that the vasculature is profoundly affected in sensitized animals. A drop of horse serum applied to the mesentery of a frog previously injected with that antigen showed pronounced capillary changes. Abell and Schenck<sup>57</sup> demonstrated marked contraction of the arteries of the ears of sensitized rabbits when they were reinjected with homologous antigen. In wheals, the common lesion of many allergic disorders, there is marked dilation and increased permeability of the capillaries.

These observations may be linked to the circumstances under which periarteritis nodosa, a rare complication, follows asthma. One possible clue resides in the fact that in the series of cases in this report, asthmatic paroxysms were usually of marked intensity. One characteristic of all forms of hypersensitivity with the exception of some bacterial types is their reversibility. After seizures of asthma or hay fever, for instance, at least in the early stages, the tissues involved appear entirely normal after the attacks cease. This is particularly true of urticaria. No matter how extensive whealing may be or how often repeated, there is complete reversibility to normal skin.

It would appear that possibly the intensity of the asthmatic attacks, or rather the intensity of the underlying mechanism that causes them, may lead to irreversible reactions in the arteries. This possibility is suggested by a similar circumstance that occurs in rheumatic fever. It is believed that the vasculature participates generally in each acute attack. The process is reversible except after intense attacks occur repeatedly when permanent rheumatic arteritis may occur. As this lesion progresses it may closely resemble periarteritis nodosa.

This transition from hypersensitive reversible reactions to nonreversible ones is typified by the Arthus phenomenon. A rabbit injected repeatedly with horse serum and tested intradermally with this antigen will at first develop a typical wheal which disappears completely. On subsequent intradermal injection a large wheal appears which subsides more slowly. Further testing induces greater whealing, then cellular induration, and finally the process is no longer reversible and tissue necrosis occurs. At this stage, necrosis of arteriolar walls appears, as pointed out by Gerlach.<sup>58</sup> The similarity of allergic processes that lose their reversibility to the Arthus phenomenon has been noted repeatedly.<sup>11, 59</sup>

The identification of most cases of periarteritis nodosa with nonatopic or intrinsic asthma is interesting in one respect. The underlying mechanism of this type of asthma is as yet unknown. One prevailing theory is that large amounts of histamine (or H-substance) is released by the tissues, and this substance causes the local lesions. The same mechanism presumably operates in atopy when antigen comes in contact with specific antibody, but as a rule the symptoms of atopic asthma are less intense than in the nonatopic type. That histamine released in excessive amounts may likewise be responsible for the lesions of periarteritis nodosa is suggested by the experiments of Bahrmann,<sup>18</sup> who produced typical arterial changes in rabbits by repeated large injections of histamine.

The relationship between periarteritis nodosa and hypersensitiveness has attracted the attention of numerous investigators, especially in the past few years. Most of these studies have been approached from a pathologic view-

point without regard to specific types of hypersensitiveness. This presentation has utilized such material in an attempted correlation with clinical allergy. Much work remains to be done to make this association complete.

#### SUMMARY

1. In 300 consecutive cases of periarteritis nodosa bronchial asthma was identified in fifty-four, or 18 per cent.

2. When differential blood counts were available, all but three of forty-seven cases of asthma (94 per cent) showed a hypereosinophilia ranging from 11 to 84 per cent, with an average of 53.5 per cent. This is in marked contrast to 151 cases without asthma in which there were but nine instances of hypereosinophilia (6 per cent) and the average eosinophile count was 2.5 per cent.

3. The association of periarteritis nodosa to the various forms of human hypersensitiveness is discussed.

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of the "blocking test" as outlined by Wiener.<sup>4</sup> For this test, 2 per cent suspensions of Rh<sub>1</sub> and Rh<sub>2</sub> cells are employed. Two drops of the unknown serum are added to two drops of the suspensions. One drop of a standard (titer 4 to 8) anti-Rh<sub>0</sub> serum, known to produce 4 plus agglutination of the test cells, is then added to the mixture. Incubation, centrifugation, and search for agglutination are subsequently carried out as in the case of the anti-Rh tests.

The following case report will serve to illustrate the sort of test tube results frequently obtained by these methods in the severer forms of erythroblastosis fetalis:

Patient 2 was a Group A, Rh-negative woman married to a Group O, Rh-positive, homozygous man. Her first child, born in 1934, was normal. The second pregnancy (1936) resulted in the stillbirth two weeks before term of an infant with intense icterus and moderate edema. The third pregnancy (1937) also resulted in the stillbirth, four weeks before term, of a baby with icterus and severe edema. In 1940, the patient again delivered a stillborn with severe edema four to six weeks before term. And in 1942, in the seventh month of pregnancy, she once more delivered a stillborn with most extensive hydrops fetalis. This woman's serum tested repeatedly from 1940 to 1942 was never shown to contain more than traces of anti-Rh agglutinins by ordinary methods. The incubation test at the present time shows only traces of agglutinins.

This Rh-negative woman, married to an Rh-positive man, had a normal first-born child. Four succeeding pregnancies resulted in infants with severe hemolytic anemia of the newborn with intense icterus and increasing and earlier fetal hydrops. Certainly her serum might have been expected to contain powerful anti-Rh agglutinins, since succeeding pregnancies resulted in greater and earlier damage to each infant. Yet repeated attempts to demonstrate anti-Rh agglutinins in the test tube never showed more than trace reactions and often were unsuccessful. Obviously in this patient and in many others with a similar history, this method of detecting these abnormal agglutinins failed.

Additional cause for dissatisfaction with the present method of testing is found in our previously reported observations<sup>3</sup> upon a serum pool in which, by the incubation method, anti-Rh agglutinins were not demonstrable. In this experiment, a measured amount of this "inactive" serum was given intravenously to an Rh<sub>1</sub>rh individual. Tests of the patient's blood after injection showed some of the red cells to be bound by inhibitor substance and therefore unagglutinable in the test tube by any other Rh<sub>0</sub> serum. At the same time the patient's serum showed the presence of free anti-Rh<sub>0</sub> agglutinins. In other words, the serum previously regarded as inactive was separated in the patient's blood stream into an inhibitor substance which attached itself to the red cells and an agglutinin which at this time was free in the serum. In vitro absorption experiments corroborated this in vivo finding: When the subject's initial cells were added to the inactive serum in a ratio of 1 or more parts of serum to 1

TABLE I  
EFFECT OF ADDING POOLED "INACTIVE" SERUM TO Rh<sub>1</sub>rh CELLS

Part of Serum	10-----1	1	1	1	1	1	1	1	1	1
Part of Blood	1-----1	2	3	4	5	6	7	8	9	10
Degree of Agglutination										
	0-----0	±	+	++	+++	++++	++++	HEMOLYSIS		

part of blood, there was no agglutination, but when an increasing concentration of red cells was tested, agglutination became more and more apparent. With a ratio of 1 part serum to 6 parts blood, agglutination was 4 plus. Beyond this concentration of red cells, hemolysis occurred. This result is illustrated in Table I.

These observations led to the examination of several anti-Rh' sera which we had found to be like those described by Race;<sup>5</sup> that is, capable of inhibiting the action of anti-Rh<sub>0</sub> sera on Rh<sub>0</sub> cells but incapable of producing agglutination when added in excess to a 2 per cent suspension of such cells. In this case, at incubator temperatures (37° C.) no demonstrable agglutination occurred,

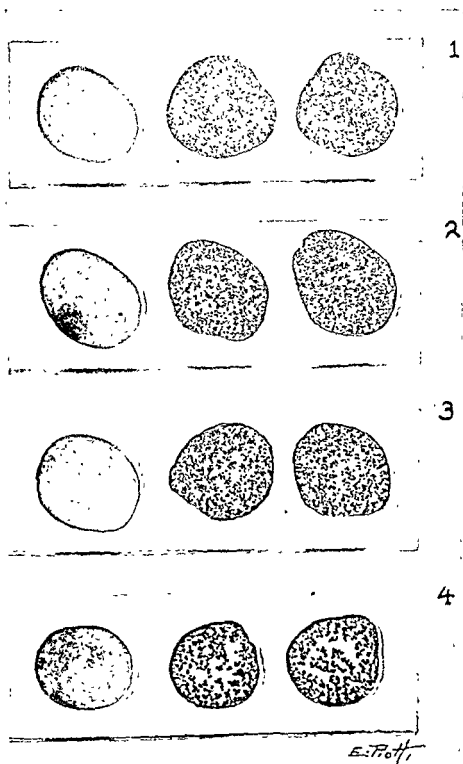


Fig. 1.—Four slides illustrating agglutination of varying strength. In each case, control, unagglutinated, Rh-negative blood is on the left, Rh<sub>0</sub> blood in the center, and Rh<sub>0</sub> blood on the right. No difference between the subtypes of Rh-positive cells is demonstrable in the final agglutination. 1, 1 plus agglutination; 2, 2 plus agglutination; 3, 3 plus agglutination; 4, 4 plus agglutination.

even after several hours, but at room ( $20^{\circ}\text{C.}$ ) and at refrigerator ( $4^{\circ}\text{C.}$ ) temperatures, agglutination and, eventually, hemolysis took place. Subsequent incubation did not break up the agglutinates, thus supporting the assumption that Rh, and not "cold," agglutinins were responsible for the reaction.

The question immediately presented itself as to whether inactive sera from mothers of erythroblastotic infants would not behave in the same way, and upon investigation this was found to be the case. That is, sera from sensitized women in which no anti-Rh agglutinins could be demonstrated by the ordinary

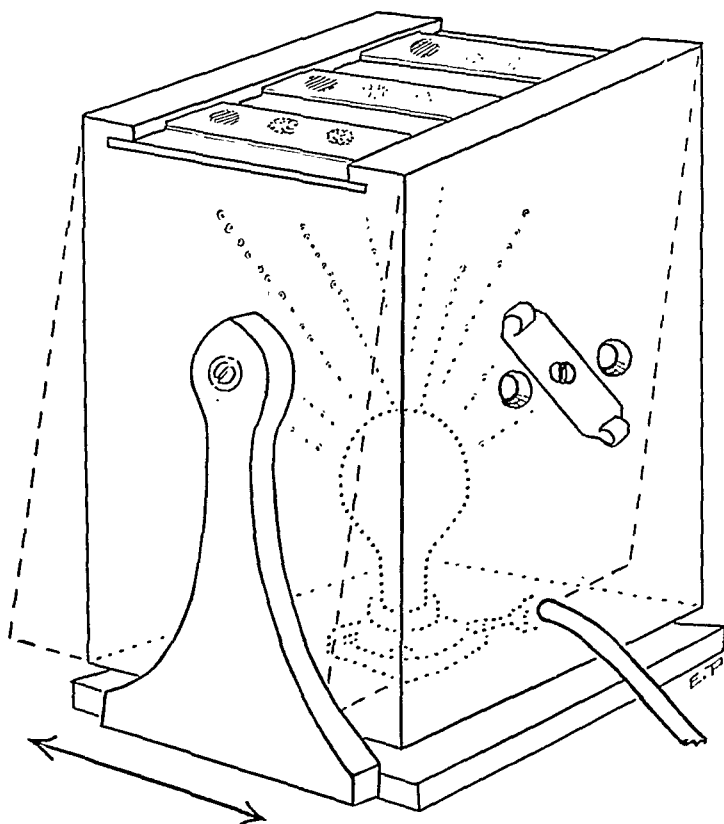


Fig. 2.—Illuminated box with ground-glass top for rocking slides and viewing agglutination. Air vents on both sides dissipate some of the heat from a 40-watt bulb and keep the ground-glass surface at about  $37^{\circ}\text{C.}$

method, were capable of agglutinating and even of hemolyzing Rh-positive cells when such cells were added in excess. Sera from Patients 1 and 2, previously described, provided two of our test cases.

The conclusion seemed justified, therefore, that certain sera contained agglutinins masked by an inhibitor substance\* and that the interference of this substance with the demonstration of agglutination was favored by dilution of agglutinin (that is, Rh-positive erythrocytes) and incubation.

These observations suggested a simple test, which has now become standard in our laboratory, for detection of anti-Rh agglutinins: Approximately 0.2 c.c.

\*The inhibitor substance is fully discussed elsewhere.<sup>2-5</sup>

of fresh, oxalated, Rh-negative, Rh<sub>2</sub> and Rh<sub>1</sub> Group O whole blood or washed cells are placed upon an ordinary slide. Cells of different specificity are used for the same reason as with the test tube method. If washed cells are used, the suspension is made up to approximately 40 to 50 per cent. With each of these is mixed 0.1 c.c. of the serum to be tested. The mixture is gently rotated or repeatedly tilted. The reaction seems to be accelerated when the slide is warmed to 37° C. or thereabouts, but it is easily performed at room temperature. Reading of the test is facilitated by holding the slide on a piece of ground glass placed over an ordinary electric light, but a mirror or white surface also serves to reflect sufficient natural or artificial light through the slide to reveal agglutination from its onset. When the serum is from a sensitized individual, usually within a minute, and certainly within three minutes, plainly visible agglutination appears in one, and usually in both, of the Rh-positive bloods. The Rh-negative blood serves as a control to eliminate errors through mistaking fibrin shreds, rouleaux formation, or nonspecific agglutination for actual Rh agglutination. Rouleaux may be broken up by adding a drop of saline and stirring it into the mixture of blood and serum. Saline does not break up agglutinates but rather makes them stand out more clearly. Fibrin is eliminated in large part by using oxalated test blood, but in the event a drop of finger blood is used for testing, the oxalate may be added to the serum. It should be emphasized that in order to obtain a satisfactory slide test, one must follow carefully the principle that a thick drop of blood is necessary. If thin suspensions are used on the slide, the results will be equivocal. This may be illustrated by the following case: Serum from Patient 2 contained no demonstrable anti-Rh agglutinins in the test tube, but the slide test was positive 4 plus. When used with increasingly thinner cell suspensions, the results shown in Table II were obtained.

TABLE II  
EFFECT OF DILUTING RED CELLS ON THE SLIDE TEST

Strength of red cell suspension	50%	40%	30%	20%
Degree of agglutination with anti-Rh serum	++++	+++	++	0

Suspensions heavier than 50 per cent may be used, but the clumps are usually so close together as to be hard to visualize. A drop of saline, of course, will overcome this difficulty. Suspensions of red cells less than 30 per cent may show agglutination, but this will vary with the type of agglutinin and its concentration in the serum. For these reasons 40 or 50 per cent suspensions of red cells have been found most satisfactory.

The test as outlined has been devised to meet the needs of a laboratory evaluating many specimens of blood serum each day. For practical clinical purposes, it is not necessary to measure the cells and serum exactly. Suitable proportions are obtained when a large drop of blood from an ordinary finger prick is mixed with approximately half as much serum. Nor is it necessary to be particularly concerned about specificity of the red cells used if the test is set up with several different specimens of Rh-positive blood. If the mother's blood is of the same group and therefore contains no isohemagglutinins against the infant's or the father's blood, it is suggested that theirs be used as the



test cells. The mother's cells, of course, may conveniently be employed as the Rh-negative control. The reaction with the infant's cells alone, if negative, against the mother's serum should not be depended upon, because it is possible in some cases that a high proportion of the baby's cells may be combined with inhibitor substance and fail to agglutinate. In cases of possible blood incompatibility between donor and recipient, the recipient's serum may be set up on a slide with the donor's fresh oxalated or citrated cells.

Until more information is available concerning the nature of the inhibitor substance and its mode of action, it is not possible to state with certainty the mechanism of the slide test. However, it seems likely that two factors, an agglutinin and an inhibitor substance (both capable of attachment to cells and both therefore presumably antibodies), exist independently in most sera. When inhibitor substance is present in nearly equivalent or stronger titer than agglutinin and a thin cell suspension is used, most of the red cells are bound by inhibitor substance, leaving so few cells to be bound by agglutinin that visible clumps cannot be formed. If, on the other hand, large numbers of red cells are used, enough erythrocytes are present both to absorb the inhibitor substance and to combine with agglutinin in sufficient number to form easily visible clumps.

We have found by the slide test method that all our sera which had been classified here and elsewhere as anti-Rh' are capable of agglutinating Rh<sub>2</sub> cells on the slide, and sera classified as anti-Rh<sub>0</sub> are capable of agglutinating Rh'/Rh' cells by this method. We have one anti-Rh'' serum which does not agglutinate Rh<sub>1</sub> or Rh' cells in this way.

Using the slide test, serum from Patient 1 gives strongly positive agglutination with Rh<sub>2</sub> cells. Therefore, although no agglutination of such cells is apparent in the test tube, the patient has strong agglutinins against such cells in her serum and the transfusion reaction resulting from the infusion of Rh<sub>2</sub> cells is readily understandable. Serum from Patient 2 also shows the presence of strong anti-Rh agglutinins on the slide, and the fact that she has given birth to children with severe erythroblastosis fetalis is no longer puzzling.

To date more than 250 slide tests have been performed in parallel with test tube tests for anti-Rh agglutinins and inhibitor substance. Of these, ninety have been on sera from mothers of erythroblastotic babies. By the usual incubation technique, only thirty-seven of these sera were found to contain anti-Rh agglutinins. The slide test was positive in all of these cases. The test for inhibitor substance in sera from sensitized individuals was in most cases positive when the usual test for anti-Rh agglutinins was negative or weak. In a few cases, however, the agglutinin and inhibitor substance present in the mother's serum appear to be so nearly balanced that only by elaborate testing, with careful control of all variables, can either be demonstrated by the test tube method.

In addition to the ninety cases mentioned above, thirty-eight of our stored sera, known to contain anti-Rh agglutinins, have been retested. The slide test was strongly positive, whereas the incubation test showed varying degrees of agglutination.

As an evidence of Rh sensitization, three methods of testing for antibodies induced by the Rh antigen have been used: namely, the slide test, the incubation test, and the test for inhibitor substance interfering with the incubation

agglutination technique. In tests of ninety consecutive cases of probable Rh sensitization the slide test confirmed the presence of antibodies in a higher percentage of cases than either of the other two tests. This is illustrated, for twenty-two of the sera tested, in Table III.

It can readily be seen that in these twenty-two cases, sensitivity was indicated by the incubation test for agglutinins in thirteen, by the "blocking test" in sixteen, and by the slide test in twenty-two. The results of the combined incubation and blocking test indicated sensitization in twenty-one of the twenty-two. It is not unlikely that if appropriate conditions are provided, test tube

TABLE III

COMPARISON OF THE SLIDE TEST WITH OTHER TESTS FOR RH SENSITIZATION

TYPE OF CELLS	SLIDE TEST			TEST TUBE TEST			"BLOCKING TEST"		
	1	2	3	4	5	6	7	8	3*
	Rh <sub>1</sub>	Rh <sub>2</sub>	rh	Rh <sub>1</sub>	Rh <sub>2</sub>	rh	Rh <sub>1</sub>	Rh <sub>2</sub>	Control
Serum 1	+++	+++	0	0	0	0	0	0	++++
2	+++	+++	0	++++	+++	0	++++	++++	++++
3	++++	++++	0	0	0	0	0	++	++++
4	++	++	0	+	++	0	++	++	++++
5	++++	++++	0	++++	++++	0	++++	++++	++++
6	++++	++++	0	++++	++	0	++++	++	++++
7	++	++	0	0	0	0	0	+	++++
8	++	±	0	++	++	0	++	+	++++
9	++++	++++	0	+	Tr	0	++++	++++	++++
10	+	++	0	+++	+	0	++++	++++	++++
11	++	+++	0	0	0	0	0	0	++++
12	+++	++	0	+++	0	0	+++	+	++++
13	±	±	0	0	0	0	++++	++++	++++
14	++	++	0	0	0	0	0	0	++++
15	+++	+++	0	++	0	0	+++	0	++++
16	++++	++++	0	++	++	0	±	++	++++
17	+	+++	0	++	+	0	++++	++++	++++
18	+++	++++	0	0	0	0	0	0	++++
19	++++	++++	0	0	0	0	0	+++	++++
20	++	+++	0	0	0	0	0	0	++++
21	+++	+++	0	+	+++	0	+	++++	++++
22	++++	++++	0	0	0	0	0	+++	++++

\*The "blocking test" described involves the mixture of Rh+ cells with unknown serum, followed by addition of a serum known to produce strong agglutination in the test tube. Therefore, 4 plus reactions in columns 7 and 8 mean no "blocking" or inhibitor substance; 1 plus to 3 plus results mean partial inhibition; and a negative result means that strong inhibitor substance is present and prevents agglutination.

methods will demonstrate antibodies in as many cases as does the slide test. Indeed, investigators carefully performing sensitive tube tests have reported the demonstration of anti-Rh agglutinins in sera of women bearing erythroblastotic babies in a higher percentage of cases than have workers using the incubation method outlined above.<sup>6, 7</sup> The "blocking test" will probably also demonstrate antibodies in a higher percentage of cases of sensitized women if the variables are so arranged as to favor inhibition. This would very likely involve the use of cells and sera of several specificities, or careful titration to demonstrate zoning, which also apparently indicates inhibitor activity. Since such procedures are laborious, the simplicity of the slide test recommends it for clinical use.

By the slide test, anti-Rh agglutinins have also been demonstrated in the sera of nine Rh-negative persons known to have been sensitized by transfusion of Rh-positive blood in whom the incubation method gave negative results.

This method has not yet received adequate trial to be proposed as a substitute for the incubation method of demonstrating anti-Rh agglutinins. Indeed, although cells of different specificity frequently react a little differently with the various sera, the reactions are usually not sufficiently clear cut on the slide for distinguishing the subgroups of Rh-positive cells. Moreover, it is essential to determine the clinical importance of the inhibitor substance, and for this purpose it seems necessary that parallel tests by the three different methods continue to be performed. For clinical work, however, the slide test does appear to possess the following advantages:

1. It appears to demonstrate Rh sensitization in a higher percentage of cases than does any other single test.
2. It more nearly pictures what is likely to occur in vivo, where a relatively large amount of agglutinin is ordinarily exposed to a smaller amount of agglutinin, both in cases of incompatible transfusion and in cases of erythroblastosis fetalis.
3. It is quickly performed with simple equipment.
4. It permits the use of testing sera heretofore considered of no value.

#### SUMMARY AND CONCLUSIONS

A simple and effective slide test is described for the demonstration of anti-Rh agglutinins in the sera of sensitized individuals, and the advantages of this method are outlined. In contrast to the incubation test, which fails to show the presence of anti-Rh agglutinins in over 50 per cent of cases chiefly because of the presence of inhibitor substances, this slide test has demonstrated agglutinins in the sera of seventy-nine of eighty women who have delivered infants with proved erythroblastosis fetalis and in nine persons who had been sensitized by transfusions of Rh-positive blood cells.

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# THE BASIC ELECTROCARDIOGRAPHIC PATTERNS IN BUNDLE BRANCH BLOCK

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**I**N A recent paper we studied the distribution of electrocardiographic potentials over the surface of the body in the normal and hypertrophied heart.<sup>1</sup> Using a similar method, we have extended our observations to cases of bundle branch block in an endeavor to correlate the electrocardiographic patterns observed with the actual spread of the impulse through the ventricles.

The concept of bundle branch block was first developed by Eppinger and Rothberger in 1909.<sup>2</sup> A few years later Carter<sup>3</sup> formulated the criteria for the diagnosis of bundle branch block in man. It was recognized at that time that there were two main types of bundle branch block patterns in the standard leads. The first type consisted of records in which the main QRS deflection of Lead I was upright. This was interpreted as right bundle branch block by Lewis and others.<sup>4</sup> The second type consisted of records in which QRS-I pointed downward. This was interpreted as left bundle branch block.

The first serious objection to this classification came from the work of Barker, Macleod, and Alexander<sup>5</sup> and from the observations of Wilson and his associates,<sup>6, 7</sup> who used unipolar and multiple precordial leads in their studies. They showed that cases of so-called right bundle branch block were actually due to disturbance of the main left bundle and that cases of so-called left bundle branch block were in reality due to interruption of the left main bundle. These concepts of Wilson are generally accepted today.

In the observations described in this paper we have accepted and extended the principles of bundle branch block enunciated by Wilson. Although we have made use of unipolar leads, my modification<sup>8</sup> of Wilson's indifferent electrode of zero potential<sup>9</sup> was used in taking the unipolar leads, and the unipolar extremity leads were taken with our technique of obtaining augmented unipolar extremity leads.<sup>8</sup> The records obtained, however, are identical with those taken according to the original technique of Wilson.<sup>9</sup>

## I. MATERIAL

For the purposes of this study we made use of a series of electrocardiograms from fifty cases of bundle branch block drawn from our files. In all these cases we had taken not only the standard leads, but also unipolar extremity leads and multiple unipolar precordial leads, using the technique mentioned.

Further, in twenty-five of these cases multiple unipolar precordial leads had also been taken from the following points on the surface of the body:

- 2 R.ics.—Electrode on right mid-clavicular line at level of second intercostal space

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- 2 m.st.—Electrode on sternum at junction of manubrium and body of sternum
- 2 L.ics.—Like 2 R.ics. but on left mid-clavicular line
- 4 R.ics.—Electrode on right mid-clavicular line at level of fourth intercostal space
- R.U.A.—Electrode on right upper abdominal wall on continuation of right mid-clavicular line, at level of lowest border of ribs
- L.u.B.—Electrode on left upper back at the base of spine of left scapula
- L.m.B.—Electrode at angle of left scapula
- L.l.B.—Electrode on left lower back on twelfth intercostal space, directly below angle of left scapula
- R.u.B.—Like L.u.B. but on right side
- R.m.B.—Like L.m.B. but on right side
- R.l.B.—Like L.l.B. but on right side

The multiple unipolar precordial leads were taken in the following way:

- V-1—Electrode at right sternal border on fourth intercostal space
- V-2—Electrode at left sternal border on fourth intercostal space
- V-3—Electrode placed midway on line drawn from the points of application of Leads V-2 and V-4
- V-4—Electrode on left mid-clavicular line at level of fifth intercostal space
- V-5—Electrode on left anterior axillary line at level of fifth intercostal space
- V-6—Electrode on left mid-axillary line at level of fifth intercostal space.

All cases in which myocardial infarction was present in addition to the bundle branch block were excluded from this series.

We also studied electrocardiograms from five cases of left ventricular hypertrophy in which there was a prolongation of the QRS interval (0.11 second or more), using the above leads.

## II. PHYSIOLOGIC CONSIDERATIONS

When heart muscle becomes activated, electrical potentials are produced, the active muscle being relatively (-) to the inactive muscle (and the inactive muscle being relatively [+] to the active). This potential difference exists at the junction between the inactive muscle and the muscle which is undergoing activation or regression, because muscle which is fully active (or inactive) has the same potential throughout, and such a region can have no effect on the electrocardiogram. As the impulse travels over the heart muscle, it may therefore be regarded as having a (+) pole in the direction toward which it is progressing and a (-) pole in the direction from which it is passing (Fig. 1). If impulses from two or more regions of the heart are traveling in the same direction, their effect will be augmented. If they are traveling in opposite directions, they will tend to counterbalance each other.

In the normal heart, the impulse travels from the subendocardial region outward to the epicardium. Theoretically both sides of the interventricular

septum are considered to be activated simultaneously,<sup>10</sup> although this probably is not so.<sup>11, 12</sup> (Whether it is or not is not important to the thesis of this paper.) However, in a case in which one of the main branches of the bundle of His is interrupted, one side of the septum is definitely activated in advance of the other, and from a theoretical point of view the electrocardiographic patterns which would be obtained from a unipolar lead overlying the surface of the left ventricle and facing the left side of the septum and from a unipolar lead overlying the surface of the right ventricle and facing the right side of the septum can be anticipated from a knowledge of the spread of the impulse. For example, in a case in which conduction through the right main branch of the bundle of His has ceased for one reason or another, the following conditions would exist (*right bundle branch block*):

1. The first region of the ventricle to be activated in such a case is the left side of the septum, and the impulse will begin to spread through the septum toward the right side. The effect of this initial electrical activity on the unipolar leads would be to cause a (+) upward deflection in the lead facing the right side of the septum and a (-) downward deflection from the left ventricular lead (Fig. 1, a).

2. Following this, the impulse travels outward through the normally activated left ventricle and at the same time completely penetrates the septum to reach its affected right ventricular surface. With the activation of the right side of the septum, the entire septum becomes fully activated and, as mentioned previously, the potential differences formerly present disappear and the deflection should return to the isoelectric line. However, since at the same time there is an impulse passing outward through the left ventricular wall, the right ventricular electrode faces the tail end of this wave and a (-) deflection is recorded instead. The impulse which travels outward through the left ventricle also serves to limit the depth of the initial downward deflection recorded by the left ventricular electrode. The left ventricular electrode, facing the oncoming wave through the left ventricle, records a (+) deflection (Fig. 1, b).

3. Once the impulse has penetrated the septum it spreads to that portion of the affected conducting system still functioning and then outward through the wall of the affected right ventricle. The potential at the left ventricular electrode (which had been recording a [+ ] deflection) tends to become isoelectric when the activation of the left ventricle is complete and the surface of the left ventricle is reached. Since, however, the right ventricle is still being activated, the left ventricular electrode faces the tail end of this wave, passing outward through the right ventricle, and the deflection becomes (-). The right ventricular electrode faces the oncoming wave in the right ventricle and records a (+) deflection (Fig. 1, c).

4. The last region of the heart to become activated is the right ventricular surface. When this occurs the (+) potential at the right ventricular electrode becomes isoelectric, as does the (-) deflection of the left ventricular electrode (Fig. 1, d).

With left bundle branch block the potentials of the right and left ventricular leads should be just the reverse (Fig. 2).

It can therefore be said that an electrode overlying the affected ventricle and facing the affected side of the interventricular septum should record an M-shaped QRS complex, and an electrode overlying the contralateral normal ventricle and facing the normal side of the septum should record a W-shaped QRS complex. Furthermore, inasmuch as conduction through the muscle of

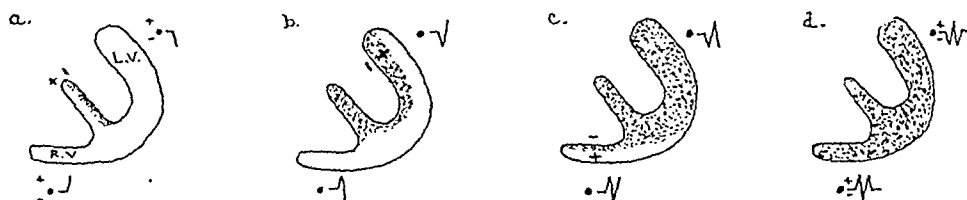


Fig. 1.

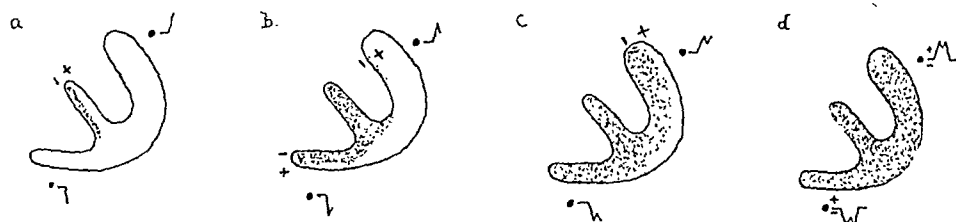


Fig. 2.

Figs. 1 and 2.—Schematic representation of the spread of the impulse through the heart in cases of bundle branch block and the basic electrocardiographic patterns which would be obtained. The stippled areas represent regions which are being or have been activated. The heavy dots represent electrodes, and the graphs next to them represent unipolar electrocardiograms which theoretically would be obtained if the heart were in the stage of activity represented in the drawings. For further details see text.

the interventricular septum is slower than through the conduction system,<sup>13</sup> it would be expected that the duration of the QRS complex, which is a measure of this, should be increased. That this actually occurs can be seen in the following examples:

1. Fig. 3 is the record of a case of right bundle branch block. Note the M-shaped QRS in the precordial Leads V-1, V-2, and V-3, which face the right side of the septum, and the W-shaped QRS in the precordial Leads V-4, V-5, and V-6, which face the normal left side of the septum.

2. Fig. 4 is the record of another case of right bundle branch block. Note the M-shaped complexes in precordial Leads V-1 and V-2 and in the unipolar leads from the second right intercostal space and the right mid-back. The W-shaped QRS can be seen in precordial Leads V-5 and V-6 and in the lead from the left mid-back, although the initial downward deflection in the precordial leads is small in this case.

3. Fig. 6 is that of a case of left bundle branch block. Note that here the M-shaped QRS is found in the leads facing the affected left ventricular side of the septum, namely, precordial Leads V-5 and V-6 and the leads from the left upper and lower back in this case. The W-shaped QRS is found, on the other hand, in those leads which face the normal right side of the septum, namely, in this case, the leads from the second intercostal space anteriorly (2 R.ies., 2 m.st., 2 L.ies.), the first precordial Lead V-1, and the right upper abdominal lead.

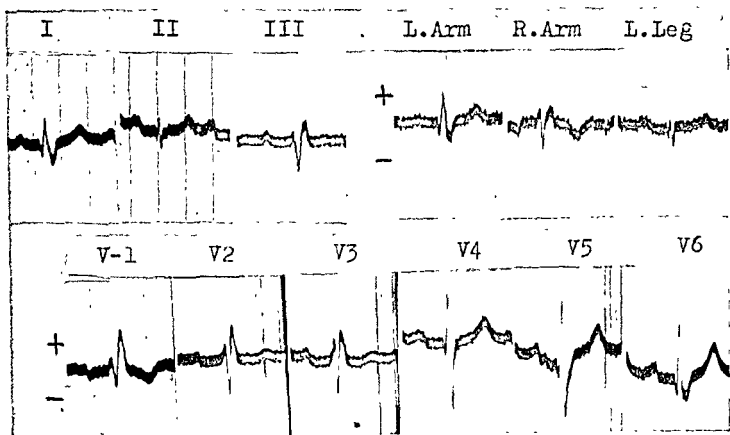


Fig. 3.—R. W., woman, 53 years of age. Right bundle branch block; heart in oblique position.

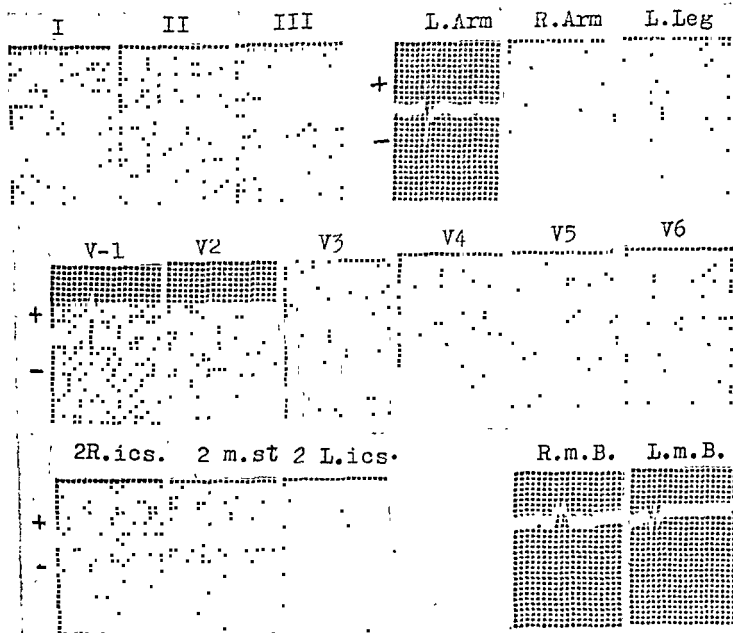


Fig. 4.—I. G., man, 85 years of age. Right bundle branch block; heart in vertical position.



4. A similar situation holds in Fig. 7 where the M-shaped complexes are found in precordial Leads V-4, V-5, and V-6 and in the lead from the left mid-back, and the W-shaped QRS complexes are found in leads from the second right interspace, the mid-sternal lead, the fourth right interspace, Lead V-1, and the right upper abdominal and right mid-back leads.

It should be noted that with left bundle branch block, the notch of the M or W does not reach or extend beyond the isoelectric line in contradistinction to the conditions theoretically anticipated and as found in cases of right bundle

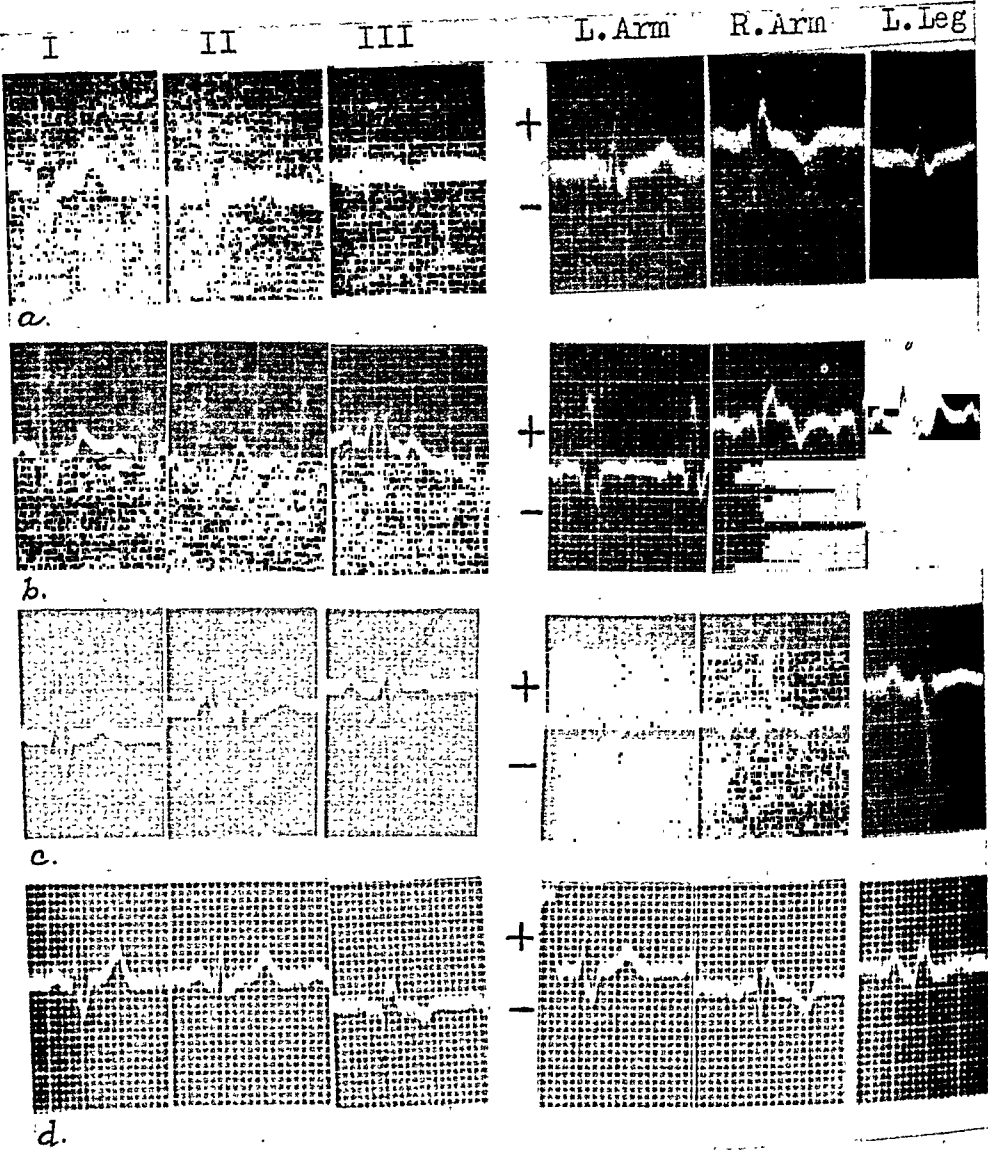


Fig. 5.—Additional cases of right bundle branch block. In a, b, and c the hearts are more or less in the oblique or horizontal position. In d the heart is more or less vertical. a, E. R., woman, 54 years of age; b, V. W., woman, 66 years of age; c, E. B., man, 51 years of age; d, R. B., woman, 67 years of age.

branch block. The explanation for this is as follows: In left bundle branch block, the initial activity of the right side of the septum is recorded as a (-) deflection at the right ventricular electrode (Fig. 2, a). The impulse traveling

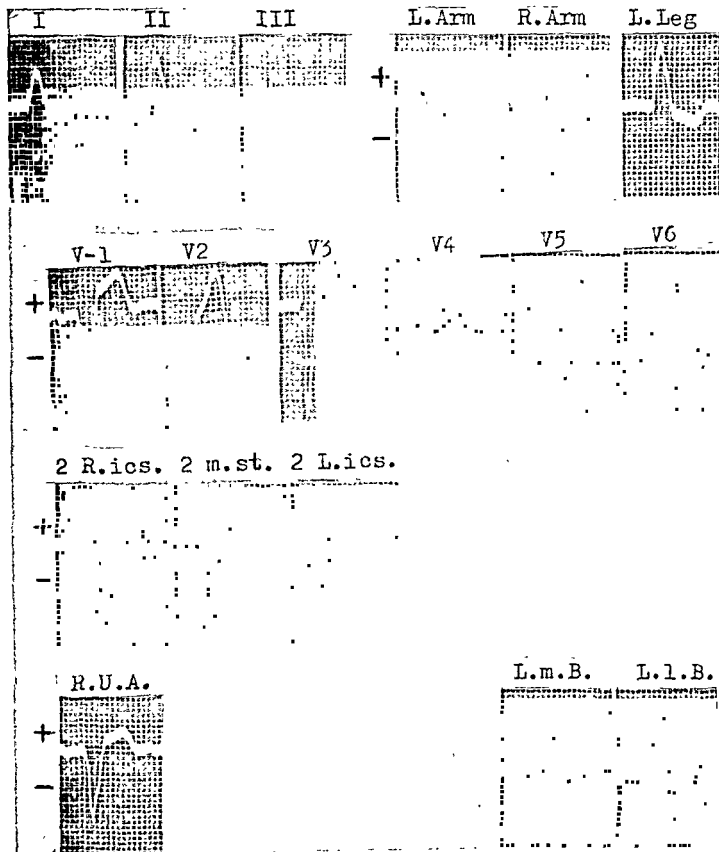


FIG. 6.—I. M., man, 88 years of age. Left bundle branch block, heart in moderately vertical position.

outward through the normal right ventricle a moment later tends to counterbalance this initial activity, but since the right ventricular wall is thinner than that of the left ventricle, the electrical activity produced within the right ventricle is weaker, and it does not interfere as much with the initial d

ward deflection as left ventricular activity does in a case of right bundle branch block. Consequently, the depth of the deflection is greater than that observed in the left ventricular lead in a case of left bundle branch block. For the same reason, after the septum has become fully activated and the sur-

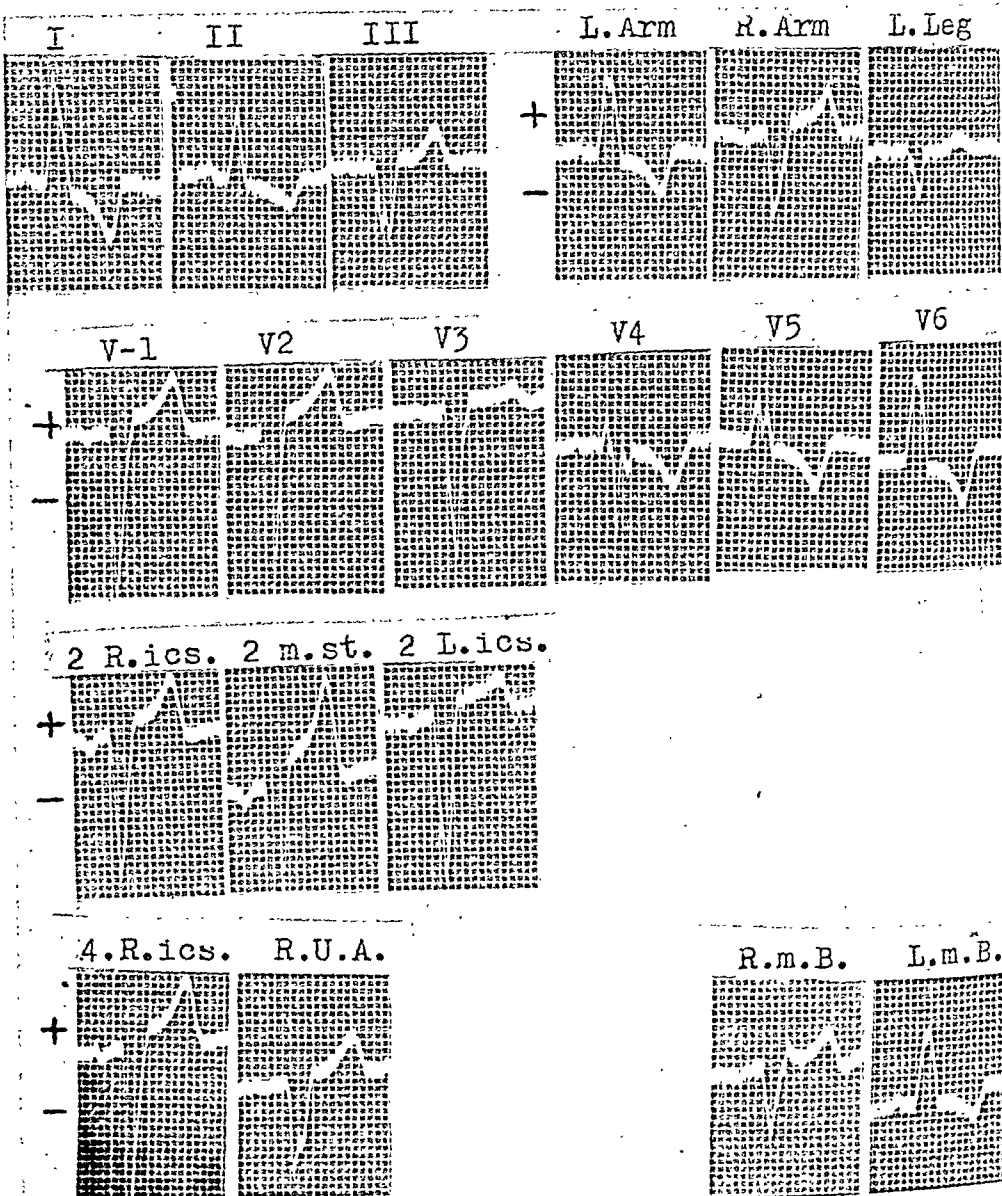


Fig. 7.—M. R., man, 63 years of age. Left bundle branch block; heart in horizontal position.

face of the right ventricle activated, the deflection tends to go to the isoelectric line, but before it can reach the isoelectric line, the impulse spreading outward through the large left ventricular wall causes the deflection to become more (-) at the right ventricular electrode (Figs. 2, b and c). The final

deflection is recorded as the surface of the affected left ventricle is activated (Fig. 2, *d*). The electrical activity recorded at the left ventricular electrode has, of course, the opposite polarity as that recorded by the right ventricular electrode.

In many cases it will be noted that instead of actual notching, which produces the M and W, the peak of QRS is just slurred. Also, QRS deflections, more complex in shape than an M or W, are occasionally observed.

### III. THE PATTERNS IN STANDARD AND UNIPOLAR EXTREMITY LEADS

The relations between standard leads and unipolar extremity leads can be summarized in the following way:<sup>14</sup> Lead I tends to resemble the left arm lead and Lead III tends to resemble the left leg lead. Since the extremities are far removed from the heart, the patterns tend to vary widely for several reasons. First, the exact area of the heart that the electrode faces is much more extensive. Second, as has been shown previously,<sup>1</sup> the extremities occupy transition zones of potential and vary greatly with the position of the heart. When the heart lies vertically, the left leg lead faces the left ventricle and the left side of the septum, and the left arm lead tends to face the endocardium of the ventricles. When the heart lies obliquely or horizontally, it is the left arm lead which faces the left ventricle and the left side of the septum, the left leg lead tending to face the epicardial surface of the right ventricle and the right side of the septum.

Thus in right bundle branch block, the following patterns may be theoretically anticipated in the unipolar extremity and standard leads:

1. *With a vertical heart*, the left leg lead should have a W-shaped QRS. The left arm lead, though facing the endocardium and therefore being predominantly (-),<sup>14</sup> also faces the left side of the septum and should also have a W-shaped QRS.

2. *With an oblique or horizontal heart*, the left arm lead should have a W-shaped QRS and the left leg lead should have an M-shaped QRS.

3. Irrespective of the position of the heart, the right arm lead tends to face the right side of the septum and should have an M-shaped QRS.

Actual unipolar extremity leads in cases of right bundle branch block correspond well to this. Fig. 4 illustrates the records in a vertical heart. Fig. 5, *a*, *b*, *c*, are cases in which the heart is more or less horizontal.

Occasionally with right bundle branch block occurring in a vertical heart, the left arm lead will tend to face the right side of the septum. The reason for this is that with a vertical heart, there is also rotation of the heart around its long axis, which causes the right ventricle to become more anterior and the apex to move posteriorly. In such a case the left arm lead will tend to have an M-shaped QRS (Fig. 5, *d*). Note that Lead I tends to resemble the left arm lead and that Lead III tends to resemble the left leg lead.<sup>1</sup>

The position of the heart is also probably responsible for the variations found in the unipolar precordial leads. For example, in Figs. 3 and 4, Leads V-1, V-2, and V-3 have an M-shaped QRS, although other of our cases showed the M-shaped QRS in precordial Leads V-1, or Leads V-1 and V-2, or even in Leads V-1, V-2, V-3 and V-4.

It should be noted that the right arm lead often does not show an initial upward deflection. In such cases, leads from the right upper chest wall anteriorly will have the characteristic deflection. This is emphasized because in left bundle branch block neither the right arm lead nor leads from the right upper anterior chest wall will have upward initial deflections.

It may also be noted that we not infrequently find records which are typical of right bundle branch block with the exception of the fact that the QRS interval does not exceed 0.1 second. The significance of such records, which we have found in normal subjects, will be discussed elsewhere.<sup>11</sup>

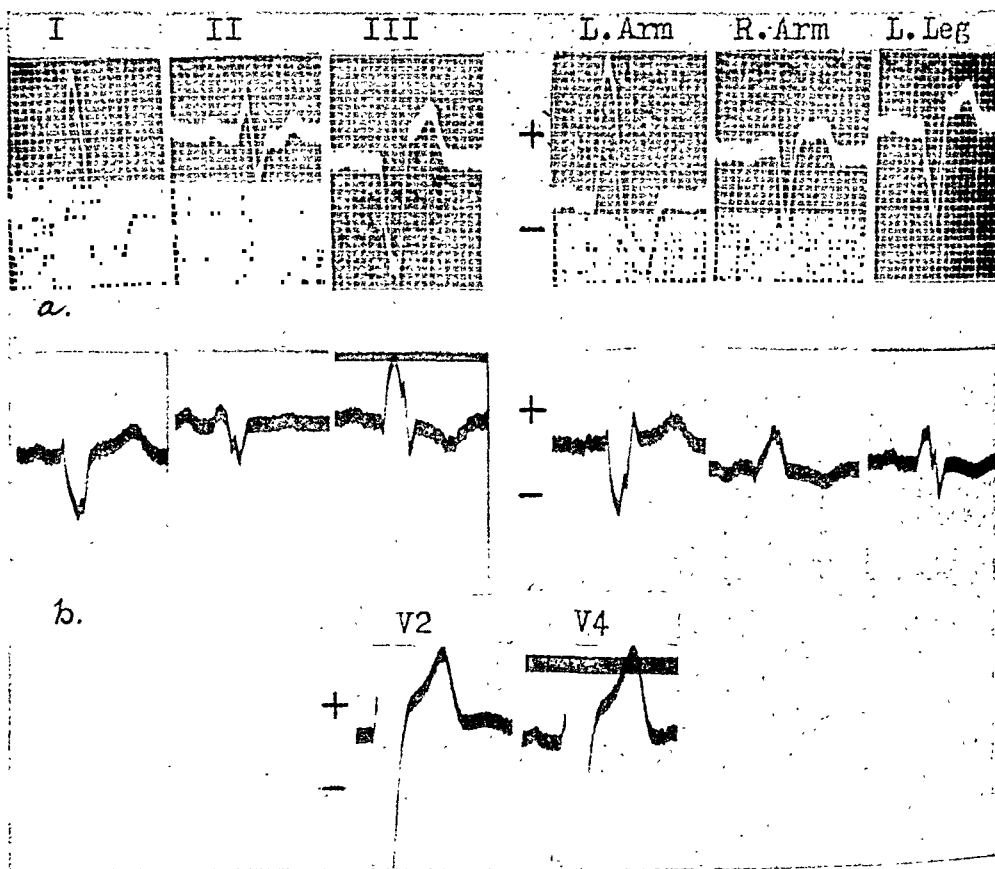


Fig. 8.—Additional cases of left bundle branch block. *a*, P. A., man, 59 years of age; heart in horizontal position. *b*, M. W., woman, 62 years of age; heart in vertical position.

In left bundle branch block *with an oblique or horizontal heart*, the left arm lead faces the left ventricle and the left side of the septum and should have an M-shaped QRS. In such a case the left leg lead tends to face the right ventricular surface and the right side of the septum and should have a W-shaped QRS. A *vertical heart* is uncommon in left bundle branch block because most of the cases have enlargement of the left ventricle which is the cause of the horizontal position of the heart. When left bundle branch block does occur with a vertical heart, however, the left leg lead will face the left side of the septum and should have an M-shaped QRS; the left arm lead

should be small but should also have an M-shaped QRS. Irrespective of the position of the heart, the right arm lead and leads from the right upper chest anteriorly should always have an initial downward deflection. This actually occurs. Fig. 6 is that of a moderately vertical heart; Figs. 7 and 8, *a*, of horizontal hearts with left bundle branch block. Note that in Fig. 7 the initial deflection of the left leg lead is upward instead of downward, as theoretically anticipated. The explanation of this is that the left leg lead does not face the right ventricle sufficiently to record the initial (-) deflection from the right side of the septum. In such a case leads farther to the right, as from the

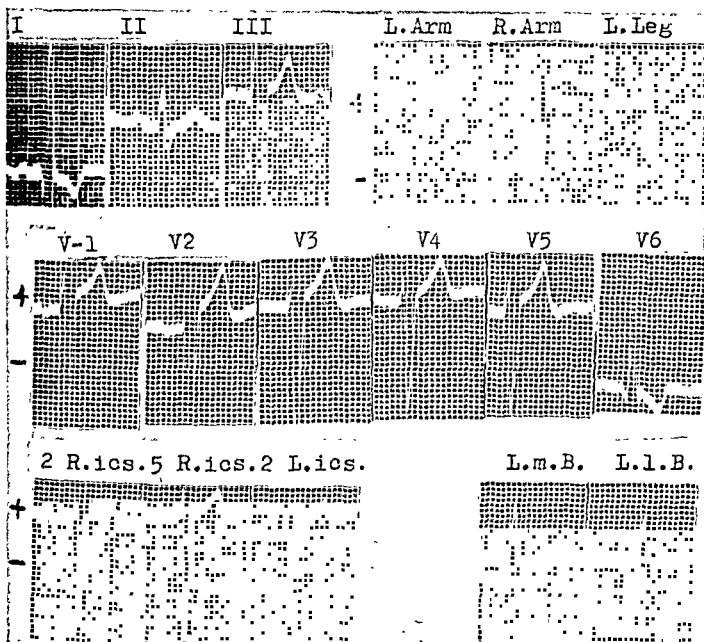


Fig. 9.—A case of left ventricular hypertrophy with prolongation of the QRS interval. J. E. man, 24 years of age, rheumatic heart disease and aortic insufficiency.

right back or right abdominal wall, often do have the initial downward deflection (Fig. 7). In Fig. 8, *a*, the initial downward deflection in the left leg lead is present. Fig. 8, *b*, is a record of a case of left bundle branch block in which the heart was markedly vertical. A similar record has been published by Wilson.<sup>10</sup>

Variations, also probably due to the position of the heart, are found in the precordial leads in cases of left bundle branch block. Usually, though not necessarily, the first precordial lead, V-1, has an initial downward deflection,

although this may also be seen in Lead V-2. The M-shaped QRS is usually not seen until the electrode is moved sufficiently to the left, usually to point V-5.

In addition to our studies on cases of bundle branch block we took multiple unipolar leads over the surface of the body in five cases of hypertensive cardiovascular disease and left ventricular hypertrophy. In these particular cases the electrocardiogram showed a prolongation of the QRS interval to either 0.11 second or more. Fig. 9 illustrates such a case, that of a 24-year-old man with rheumatic heart disease, aortic insufficiency, and massive enlargement of the left ventricle. Note that although the pattern superficially resembles that of left bundle branch block, leads from the right upper portion of the body have an initial upward deflection, and leads from the left side of the body have an initial downward deflection. The pattern is therefore similar to that of a normal horizontal heart<sup>1</sup> or to cases of right bundle branch block, insofar as the initial deflection is concerned, rather than left bundle branch block. The characteristic M- and W-shaped patterns of bundle branch are not seen. The prolonged QRS interval in such a case can be explained by the massive size of the patient's heart and the fact that it takes the impulse a longer time to penetrate the hypertrophied left ventricle than a normal heart. A similar prolongation of the QRS may be seen in cases where the right ventricle is markedly hypertrophied.

The T wave patterns observed in cases of left and right bundle branch block should be discussed briefly. An M-shaped QRS usually has a (-) T and W-shaped QRS an upward (+) T, although exceptions to this are common. These T wave patterns are due to the altered paths over which the impulse spreads and are not due to myocardial damage.<sup>10</sup>

#### IV. CONCLUSIONS

When unipolar leads are used, the electrocardiographic patterns observed in cases of bundle branch block may be directly correlated with the actual spread of the impulse through the ventricles. On the basis of theoretical considerations and actual electrocardiographic observations, the following are the basic electrocardiographic patterns in cases of bundle branch block:

1. An M-shaped QRS complex in unipolar leads overlying or facing the affected ventricle and the affected side of the interventricular septum.

2. A W-shaped QRS complex from unipolar leads overlying or facing the contralateral normal ventricle and the normal side of the septum.

3. Prolongation of the QRS interval to 0.11 second or longer.

4. A (-) T wave with the M-shaped QRS and a (+) T with the W-shaped QRS, although exceptions to this are frequent.

5. The patterns observed with unipolar extremity and standard leads depend on the position of the heart. When the heart is vertical, the left leg lead will face the left ventricle and the left side of the septum; when the heart is oblique or horizontal, the left arm lead will face the left ventricle and left side of the septum, the left leg lead now tending to face the right ventricle and the right side of the septum.

In this paper we also demonstrated the fact that *not* every case in which the QRS interval exceeds 0.11 second is necessarily due to bundle branch block but may be explained by the fact that the impulse requires a longer time to penetrate a greatly hypertrophied ventricle than it normally needs.

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# THE PRODUCTION OF BACTERICIDAL ACTIVITY IN COD-LIVER OIL BY OXIDATION

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THE Council on Pharmacy and Chemistry of the American Medical Association, in 1943,<sup>1</sup> conducted a thorough review of the literature appertaining to the value of cod-liver oil as a medicinal agent promoting the healing of wounds following topical application. Most of the controversy centered about the virtues of the agent depending upon the vitamin content of the oil. The Council concluded, at that time, that the evidence available did not warrant the acceptance of cod-liver oil preparations for the topical treatment of wounds.

Reports by Harris, Bunker, and Milas (1932)<sup>2, 3</sup> Löhr and Treusch (1934),<sup>4</sup> Stevens (1936),<sup>5</sup> Nélis (1939),<sup>6</sup> Lichtenstein (1939),<sup>7</sup> and Worley (1940)<sup>8</sup> indicate that cod-liver oil elicits bactericidal activity under various conditions. However, Kummell and Jensen (1936)<sup>9</sup> and Driver, Binkley, and Sullivan (1938)<sup>10</sup> failed to observe bactericidal activity in their preparations. These contradictory data have led us to investigate this aspect of the question. After evaluating the data available, it appears that so-called old or rancid cod-liver oils are usually bactericidal. Furthermore, treatment by heat, aeration, or ultraviolet irradiation in shallow, open dishes results in a preparation possessing bactericidal activity. Fresh cod-liver oils are rarely bactericidal. These observations suggest that the substances lethal to microorganisms are produced by the interaction of the oil with oxygen. While this was undoubtedly appreciated by earlier workers, the importance of the initial oxidation has not been recognized as the basic reaction leading to the synthesis of substances possessing bactericidal activity.

According to Hilditch (1941)<sup>11</sup> 85 to 89 per cent of cod-liver oil consists of unsaturated fats. Such of these fats as contain the ethylene linkage arranged in the highly reactive 1-4 conjugated system of double bonds, —CH=CH—CH=CH—, are known to undergo autoxidation by atmospheric oxygen and may initiate chain reactions.

The presence of naturally occurring antioxidants (Olcott and Mattill 1941<sup>12</sup>) in fats retards and may inhibit the oxidation of the unsaturated fats. Buxton (1942)<sup>13</sup> further regarded the presence or absence of antioxidants as factors in the destruction of vitamin A. Buxton's procedure for the removal of these antioxidants from cod-liver oil has been followed in this study. The development of bactericidal properties by these oils subsequent to oxidation by atmospheric oxygen is studied in this investigation.

*Materials and Methods.*—Commercial U. S. P. cod-liver oil was used in these experiments. After adding one part of a nonpolar solvent such as benzene,

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chloroform, or hexane to two or three parts of oil, five parts of this mixture were treated by stirring with one part of activated Norit for thirty minutes to an hour at room temperature. Following filtration, the solvent was distilled off at reduced pressure. This treatment removed the antioxidants, and the residual oil readily underwent oxidation when exposed to atmospheric oxygen.

The rate of production of peroxides and aldehydes was determined by the methods of Lea (1931).<sup>13</sup>

The bacteriologic tests were made using plates of infusion agar heavily inoculated with *Staphylococcus aureus*, *Bacillus subtilis*, or *Escherichia coli*. The oils were tested by direct contact on the agar using filter paper disks. The

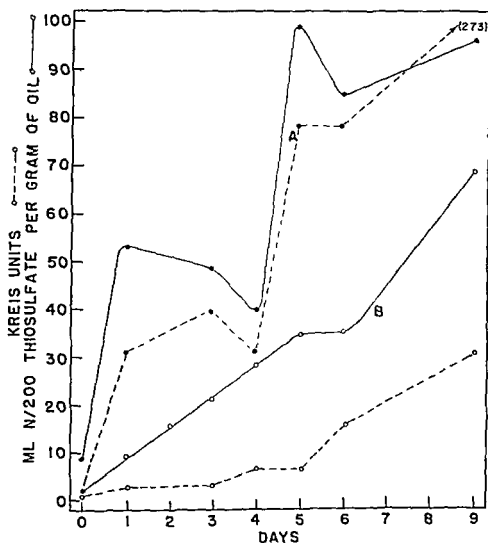


Fig. 1.—Illustrating the rate of peroxide and aldehyde formation in (B) a specimen of U. S. P. cod-liver oil and in (A) the same oil after extraction with activated Norit to remove the antioxidants in commercial cod-liver oil. The irregularity of these curves would indicate that the observed values are the summation of several reactions. The solid lines represent the peroxide values, while the dashed lines represent those of the aldehydes. (A and B are the specimens A and B of Table 1.)

vapors of the oils were tested by pouring 5 to 8 c.c. of oil into the bottom of Petri plates and inverting a second plate bottom containing the inoculated agar over the oil. Cultures and oils were incubated at 38° C. and read at twelve and twenty-four hours.

**Experimental.**—When the untreated U. S. P. cod-liver oils were tested for bactericidal activity, it was found that they were not bactericidal. After extracting the antioxidants from the oils, the preparations exhibited bactericidal properties by direct contact and by the vapors emanating from the preparations following exposure to atmospheric oxygen. Dishes of the oils were exposed to

atmospheric oxygen at 38° C. and tested daily for bactericidal activity and for the formation of peroxides and aldehydes. Adsorbed oils rapidly became bactericidal and remained so indefinitely. In contrast, the fresh U. S. P. oils did not elicit bactericidal effect until they had been exposed for at least five days.

The rate of formation of peroxide is shown in Fig. 1 in a specimen of fresh U. S. P. cod-liver oil and in another specimen of the same oil from which the antioxidants had been removed. It is apparent that the rates of oxidation differ greatly in these instances. The extracted oil was bactericidal to the three organisms testing within the first twelve hours of incubation at 38° C. In contrast, the original oil oxidized comparatively slowly and did not become bactericidal until the sixth day of incubation. While the peroxide oxygen values, expressed in cubic centimeters of N/200 thiosulfate, can only be considered as semiquantitative, because of the complex reactions taking place (Milas, 1941<sup>14</sup>), the values do indicate large increases in peroxide and the length of time required for an oil to undergo auto-oxidation after it has been exposed to the air. In Table I is shown the increase in aldehydes in these same oil specimens. The Kreis reaction for aldehydes is essentially a qualitative test. It was modified as follows to yield relative quantitative values: the reactants produced a pink color. An ethereal solution of phloroglucinol was added to the oil dissolved in chloroform, and the mixture was acidified with HCl. The color intensity was compared with an arbitrary set of standard tubes containing varying concentrations of  $\text{KMnO}_4$ . Tube 1 was designated as 1 unit; Tube 2 contained 2.5 times the concentration of Tube 1, etc.; Tube 5 contained 39 Kreis units of color. As the reaction proceeded to exceed the color intensity of Standard Tube 5, the oil was diluted with chloroform and tested. The number of units was then multiplied by the proper dilution factor to obtain the relative quantity of aldehyde in the specimen.

The data presented in Fig. 1 and Table I show the rate of peroxide formation and aldehyde production to be much more rapid in the case of the oils from which the antioxidants have been extracted than in the instance of the original U. S. P. cod-liver oil.

TABLE I

THE RELATIVE QUANTITIES OF ALDEHYDES PRODUCED DURING OXIDATION OF COD-LIVER OIL AS INDICATED BY THE KREIS REACTION (A AND B ARE A AND B OF CHART 1)

TIME (DAYS)	ADSORBED OIL A (KREIS UNITS PER C.C.)	OIL B (KREIS UNITS PER C.C.)
0	2.5	1
1	31.2	2.5
3	39	2.5
4	31.2	6.25
5	78	6.25
6	78	15.6
9	273	31.25

*The Effect of an Antioxidant.*—A specimen of extracted cod-liver oil was divided into three portions: A, B, and C. Hydroquinone was added to C in a ratio of 1 to 1,000. The three specimens were aerated with oxygen at 80° C. and the rate of peroxide formation determined. The rate of peroxide formation was retarded in C. After two and one-half hours, hydroquinone was added to B in a ratio of 1 to 1,000. The addition of the antioxidant to specimen B did not

alter the rate of peroxide formation once the reaction was underway (Fig. 2). The rate of aldehyde production was determined and was found to be comparable with that observed in Fig. 1. No aldehyde was formed in specimen C. After heating for four hours, the three specimens were tested for bactericidal activity. A and B were strongly bactericidal. C exhibited no bactericidal activity.

*The Chemical Nature of the Bactericidal Substance.*—Since the bactericidal effect was present both in the oil and in the vapors given off by the oil, it was evident that at least one bactericidal substance formed had a low boiling point. Testing these vapors with acidified starch-iodide and Schiff's reagent showed

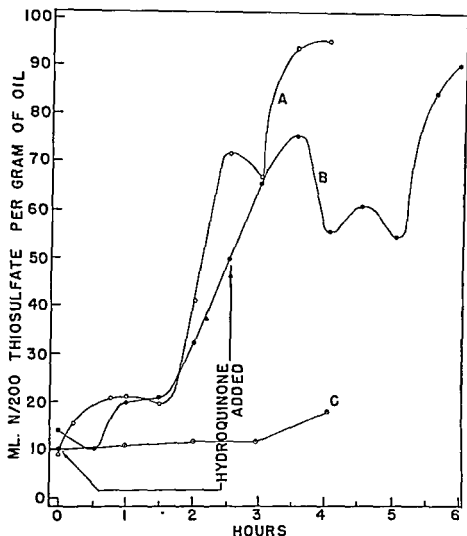


Fig. 2.—A lot of extracted cod-liver oil was divided into three portions, A, B, and C, and heated to 80° C. while oxygen was bubbled through each specimen.

Hydroquinone, an antioxidant, was added to C in a ratio of 1 to 1,000 before heating. After heating for 2½ hours, hydroquinone was added to B in the same proportions. C did not undergo oxidation, while A and B oxidized rapidly. Once oxidation had begun, the reaction was not suppressed by the antioxidant, Curve B.

them to contain both peroxides and aldehydes. Vapors from fresh U. S. P. cod-liver oil were not bactericidal and failed to give positive tests for either aldehydes or peroxides. After exposing fresh U. S. P. cod-liver oil to atmospheric oxygen for at least five days, the vapors became bactericidal and gave positive reactions for both peroxides and aldehydes. Specimens of extracted oils became dark in color and viscous when incubated at 38° C. for several months. The oils and their vapors were strongly bactericidal. The oils gave positive tests for both aldehydes and peroxides; the vapors, however, gave positive reactions for aldehydes only. These vapors contained no oxidizing substances, but they continued to have bactericidal properties and possessed an acid odor resembling

acrolein. It has not been possible to collect enough of the volatile aldehyde for chemical identification.

If the oil was heated on an open Bunsen flame, a volatile distillate could be condensed at freezing bath temperatures. These distillates were fractionated in a Vigreux column, and a clear, colorless liquid boiling at  $52^{\circ}\text{C}$ . was collected. This substance reduced bromine water and  $\text{KMnO}_4$ , was strongly bactericidal, gave a positive Schiff's test for aldehyde, and reacted with dinitrophenylhydrazine to give a compound melting at  $165^{\circ}\text{C}$ . This compound gave a mixed melting point of  $165^{\circ}\text{C}$ . with the dinitrophenylhydrazone of acrolein to indicate definitely that the distillate isolated from the heated cod-liver oil was acrolein. Obviously, it does not follow that the aldehyde occurring in the vapors of cod-liver

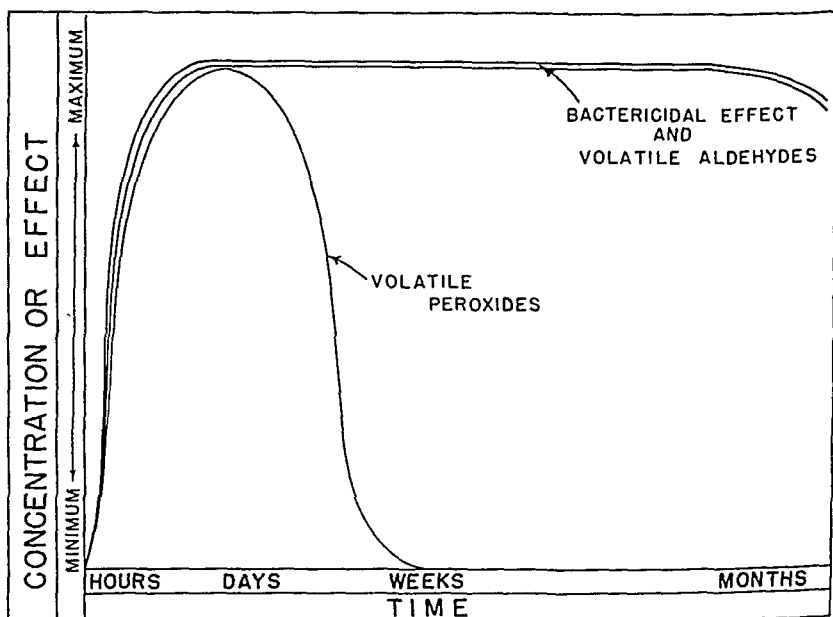


Fig. 3.—A graphic representation of the rapid and persistent production of volatile aldehydes and the acquisition of bactericidal activity by the vapors over cod-liver oil which has been treated with antioxidants. During the first days and weeks the vapors contained bactericidal activity although the substances ultimately disappeared although the vapors continued to give a positive reaction for aldehydes.

oil when exposed to oxygen at  $38^{\circ}\text{C}$ . is acrolein, since acrolein results from the pyrolysis of fats in general (Egloff and Morrell, 1932<sup>15</sup> and Vernon and Ross, 1936<sup>16</sup>). The bactericidal activity of acrolein was demonstrated by Koch and Fuchs as early as 1899<sup>17</sup> and again by Berthelot (1922)<sup>18</sup> and Vollrath and co-workers (1937).<sup>19</sup>

#### DISCUSSION

The results of this investigation would indicate that the bactericidal activity acquired by cod-liver oil is dependent upon auto-oxidation. The exact chemical nature of the bactericidal substance or substances has not been determined, but one of these substances is an aldehyde and observations indicate that the substance may be acrolein. Servias (1903)<sup>20</sup> and Dunlap and Shenk (1903)<sup>21</sup> have observed volatile aldehydes with an acrolein-like odor formed in rancid cod-liver

and linseed oils. Salway (1916)<sup>22</sup> isolated and identified acrolein upon oxidation of linseed oil and linolenic acid. Certain volatile, saturated aldehydes do, however, likewise possess bactericidal activity (Tilley and Schaffer, 1926<sup>23</sup>). Harris and associates (1932),<sup>2,3</sup> Stevens (1936),<sup>5</sup> and Lichtenstein (1939)<sup>7</sup> suggested that the bactericidal activity resided in the volatile peroxides found during the oxidation of cod-liver oil. Evidence presented by us shows volatile aldehydes to be present whenever the vapors over cod-liver oil possess bactericidal activity. Peroxides, however, cannot always be demonstrated in these vapors (see Fig. 3). Nélis and Thomas (1939)<sup>6</sup> and Worley (1940)<sup>8</sup> suggest that the bactericidal agents occurring in cod-liver oil are probably aldehydes.

Cod-liver oil is an effective mechanical covering for superficial lesions. Since an oil from which the antioxidants have been adsorbed produces bactericidal substances rapidly, this material suggests itself as a unique medicinal in the topical treatment of superficial wounds. This aspect of the problem is being studied especially in regard to burns and granulating surfaces. The report of the Council of Pharmacy and Chemistry of the American Medical Association records no untoward reactions following the topical use of cod-liver oil.

It is doubtful that the vitamin content of an oil contributes to its bactericidal activity. The volatile substances in the vapors from cod-liver oils would indicate that these substances have a low molecular weight.

#### SUMMARY

Fresh U. S. P. cod-liver oil has no bactericidal activity. Cod-liver oil acquires bactericidal activity during oxidation. This process is accelerated when the natural antioxidants in the oils are adsorbed by Norit and can be inhibited by the addition of the antioxidant, hydroquinone. Aldehydes are always present in the vapors given off by cod-liver oil when these vapors acquire bactericidal activity. It is suggested that acrolein, a bactericidal substance, may be produced by the auto-oxidation of cod-liver oil.

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## THE SEROLOGIC DIAGNOSIS OF ENDEMIC TYPHUS

### I. THE USE OF SPECIALLY PREPARED RICKETTSIAL SUSPENSIONS AND COMMERCIAL TYPHUS VACCINES AS ANTIGENS IN THE COMPLEMENT FIXATION TEST

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COMPLEMENT fixation in typhus was investigated by Castaneda,<sup>1</sup> who showed in 1936 that sera from human cases of the Mexican disease and from guinea pigs infected with the European strain of virus contained complement-fixing antibodies. Confirmation and extension of these observations were reported in 1941 by Bengtson,<sup>2</sup> who approached the problem with a view to determining the diagnostic value of the test. In this study sera from human cases of endemic typhus and from guinea pigs recovered from infections with endemic typhus were employed; the specificity of the reaction was determined with sera from cases of Rocky Mountain spotted fever and "Q" fever; normal human and guinea pig sera were used as controls. More recently, other workers, among them Reynolds and Pollard,<sup>3</sup> have reported on the utility and specificity of the test as a diagnostic procedure.

Antigens, in the case of Bengtson's work, were specially prepared from the inoculated yolk sac of the developing chick embryo following the method of Cox<sup>4</sup> and from the lungs of mice infected intranasally by the technique of Castaneda.<sup>5</sup> Reynolds and Pollard, on the other hand, used commercially prepared epidemic typhus vaccine as antigen. This vaccine was prepared from infected chick embryos and represented the product of a single manufacturer.

*Purpose of This Study.*—The purpose of this study was to determine the relative value of specially prepared rickettsial suspensions and commercially processed typhus vaccines, from a variety of sources, as antigens in the complement fixation test for typhus. At the outset, it was recognized that specially prepared antigens should prove satisfactory, but the fact that most laboratories were not in position to produce these for their own use and that they were unobtainable from commercial sources gave point to the investigation of vaccines as possible antigens as these were available on the open market.

*Materials.*—Specially prepared antigens made from the endemic type virus were furnished by the National Institute of Health‡ and Sharp and Dohme;§ the latter also provided a similar antigen of the epidemic type. The vaccines used were all prepared from the epidemic type virus and were manufactured by Lederle Laboratories, Inc.,|| Parke, Davis & Co.,¶ and Eli Lilly and Co. The standard serum used in titrating the antigens and vaccines was obtained from guinea pigs which had recovered from infections with endemic typhus or from active human cases of the disease.

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§By Dr. Florence Fitzpatrick, through the courtesy of Dr. B. Hampill.

||By Dr. H. E. Cox.

¶By Dr. F. D. Stimpert.



*The Test.*—The classical hemolytic system of sheep cells, guinea pig complement, and rabbit anti-sheep cell amboceptor was employed. In determining the antigenic titers of the specially prepared rickettsial suspensions or the vaccines, dilutions were first made covering the range 1:4 to 1:128. This was done by adding 0.1 c.c. of antigen to 0.3 c.c. saline in the first tube and transferring 0.2 c.c. of this dilution to the next tube which already contained 0.2 c.c. of saline. This was repeated through the remaining tubes of the series with 0.2 c.c. being discarded from the final tube. To all tubes, 0.2 c.c. of standard serum (diluted 1:8) was then added, followed by 0.2 c.c. of complement (2 units). The tubes were next incubated one hour at 37° C., after which 0.4 c.c. sensitized sheep cells was added. The sensitized sheep cell suspension was made by combining equal amounts of a 2 per cent sheep cell suspension and rabbit anti-sheep cell amboceptor which had been titrated to contain 2 units in 0.2 c.c. The tubes were then reincubated for one hour, placed in the icebox overnight, and read the next morning. The amount of fixation was estimated as 4+ (complete), 3+, 2+, 1+, ± (trace), and 0 (none). The titer was recorded as the highest dilution showing 3+ or 4+ fixation.

A control was always set up with each titration to establish the fact that the antigen was not anticomplementary. This consisted of 0.4 c.c. antigen (1:4), 0.2 c.c. complement (2 units), and 0.4 c.c. sensitized sheep cells.

*Results.*—The results obtained in the titration of specially prepared rickettsial antigens, when the tests were run at 37° C. with one hour allowed for fixation, are shown in Table I. This table also shows the results obtained on repeated titrations of these suspensions at varying ages.

TABLE I  
SPECIALLY PREPARED RICKETTSIAL ANTIGENS TITRATED WITH WATER BATH FIXATION FOR ONE HOUR

SOURCE OF ANTIGEN	STRAIN	DATE TESTED	ANTIGEN DILUTION						USEFUL DILUTION (4 UNITS)
			1:4	1:8	1:16	1:32	1:64	1:128	
National Institute of Health	Endemic 58	2/ 7/44	4	4	4	4	4	2	1:16
		3/20/44	4	4	4	4	4	1	1:16
		6/20/44	4	4	4	3	0	0	1:8
	Endemic 82	3/20/44	4	4	4	4	4	2	1:16
		6/20/44	4	4	4	4	4-	0	1:16
		9/8/44	4	4	4	2	0	0	1:4
		11/28/44	4	4	4-	2	0	0	1:4
	Endemic 84, Lot 3	6/20/44	4	4	4	4	4-	0	1:16
		9/ 8/44	4	4	4	4	0	0	1:8
	Endemic 84, Lot 4	6/20/44	4	4	4	4	4	0	1:16
Sharp & Dohme, Inc.	Endemic	3/20/44	4	4	4	4	4	4	1:32
		11/28/44	4	4	4	4	4	3	1:32
	Epidemic 1 2	11/28/44 11/28/44	4 4	2 4	0 2	0 0	0 0	0 0	1:1 1:2

From Table I it appears that all of the specially prepared antigens were suitable for use in complement fixation tests and that when freshly made could be used in dilutions of 1:16 or 1:32, that is, using four units in the test, the unit being the smallest amount which gave complete fixation with the standard serum. From Table I it also appears that there was some loss in antigenicity

with the lapse of time though the suspensions were still useful from four to eight months after being initially titrated.

Attention was next directed to a similar investigation of commercially prepared typhus vaccines. For this part of the study, vaccines of various lots representing the finished product of different manufacturers were obtained. These vaccines were all made from the epidemic type virus and the results of their titrations are shown in Table II

TABLE II  
RESULTS OF EPIDEMIC TYPHUS VACCINE TITRATION WITH WATER BATH FIXATION FOR ONE HOUR

SOURCE OF VACCINE	LOT NUMBER	DATE MADE	DATE TESTED	VACCINE DILUTION						USEFUL
Parke, Davis & Co.	098792 A									
			11/ 3/44	4	4	1	0	0	0	1:2
	098793 A		9/21/44	4	4	3	tr	0	0	1:4
			11/ 3/44	4	4	3	0	0	0	1:4
	098794 A		9/21/44	4	4-	tr	0	0	0	1:2
			11/ 3/44	4	3	0	0	0	0	1:2
Lederle Laboratories, Inc.	098795 A		9/21/44	4	4	3	0	0	0	1:4
			11/ 3/44	4	4	2	0	0	0	1:2
	285 H 719	4/25/44	9/ 8/44	4	4-	3	0	0	0	1:4
	721	4/26/44	9/ 9/44	4	4	4	1	0	0	1:4
	725	3 /3	9/ 8/44	4	4	3-	0	0	0	1:4
		and 23/44								
	726	3/ 3 and 22/44	9/ 8/44	4	4-	0	0	0	0	1:2
	739	2/21 and 4/ 5/44	9/ 8/44	4	4-	2	0	0	0	1:2
	43	5/ 1/44	9/ 8/44	4	4-	3	0	0	0	1:4
	45	5/ 2/44	9/ 8/44	4	4-	3	0	0	0	1:4
Eli Lilly and Co.	50	5/ 8/44	9/ 8/44	4	4-	0	0	0	0	1:2
	7084-319035		2/ 7/44	0	0	0	0	0	0	None

The results recorded in Table II indicate that commercially processed typhus vaccines made from the epidemic virus by two manufacturers and representing twelve different lots were all satisfactory for use as antigens in the complement fixation test. Not all the vaccines could be used as antigens in diagnostic tests in the same dilutions, however, as some evidently were suitable only when diluted 1:2, while others could be diluted 1:4. In contrast to these results the product of one company was entirely unsatisfactory as its vaccine was not capable of fixing complement in any dilution in which it was not anticomplementary. Incidentally, it is of interest to note that none of the other vaccines were anticomplementary and that the single unsatisfactory product encountered was that with which Reynolds and Pollard<sup>3</sup> reported obtaining good results. Failure with this product has been reported to us in a personal communication by other investigators.

From Table II it is also seen that the keeping qualities of vaccines to be used as antigens were good. In only one instance was there an apparent loss of antigenicity in the vaccine as manifested by a drop in the dilution which could be made when using it as an antigen.

In contrasting commercial vaccines with specially prepared suspensions of rickettsias as antigens, the advantage lies with the latter, as might be anticipated. This is indicated by the higher antigenic titers obtained and may be accounted for by (1) the greater concentration of the suspensions, (2) the fact that they were prepared from endemic virus, and (3) the use of endemic virus antiserum in carrying out the antigenic titrations.

#### CONCLUSIONS

1. Specially prepared chick embryo rickettsial suspensions made from epidemic or endemic typhus virus are satisfactory antigens for the complement fixation test.

2. Commercially processed epidemic typhus vaccines representing twelve lots of manufacture were found satisfactory as antigens in complement fixation.

3. No anticomplementary factor was encountered in any of the commercial vaccines shown to be satisfactory as antigens.

4. Typhus vaccine is sufficiently antigenic to be diluted 1:2 or 1:4 in complement fixation.

5. One vaccine was not antigenic in any dilution in which it was not anti-complementary.

6. Typhus vaccines have been found to be satisfactory antigens in complement fixation tests at least for six months after manufacture.

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## EFFECT OF PENICILLIN ON EXPERIMENTALLY PRODUCED PLAGUE IN GUINEA PIGS

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### INTRODUCTION

SINCE 1910 plague has been present in the northern portion of the Hamakua Coast area of the Island of Hawaii, the largest of the islands comprising the Territory of Hawaii.<sup>1</sup> A number of rats have been found to be infected with the disease each year, and there has been a continued but sporadic occurrence of human cases of bubonic or pneumonic plague. One hundred and eleven human beings have been reported to the health department as having plague during the thirty-five years since the disease first appeared in this district. The maximum number of cases reported for any one year has been twelve. During five yearly intervals no cases were reported.

Plague has occurred in this area more consistently and with greater frequency than in any other area in the United States during recent decades. The mean case rate per 100,000 in the Hamakua area, with a population of approximately 10,000, has been 31.7. In the San Francisco outbreak of 1907 and 1908 the rate was approximately 20 per 100,000 for the two-year period. The rate in the New Orleans outbreak of 1914-1915 was 8.5. Of the patients in the Hamakua area having plague, 110 have died, giving an extraordinary mortality percentage of 99.1. In the San Francisco outbreak seventy-eight of 160 persons contracting the disease died, a mortality of 49 per cent. Of the thirty-one cases in the New Orleans outbreak, ten, or 32 per cent, died. A comparison of plague in these three areas is shown in Table I.

The disease occurs on the Island of Hawaii endemically in rats with an occasional epizootic upswing. Cases in man are sporadic, but the relatively high incidence and high death rate indicate the importance of using this area for studies of plague.

The effect of penicillin on *Pasteurella pestis* naturally was of acute concern, with the hope that this drug might possibly be more effective in treating individuals with plague than any other available medication. The presence of a considerable number of military personnel in the Territory has caused the Armed Forces to be intimately concerned with methods for the suppression of plague here. A protocol for an experiment with penicillin in the treatment of guinea pigs inoculated with plague organisms was prepared by the health department and was submitted to the Army Medical School through the Surgeon of the Central Pacific Base Command. Upon approval of the protocol, sufficient sodium salt of penicillin‡ to conduct the experiment at the Plague Laboratory of the Board of Health at Honokaa, Hawaii, was supplied by the United States

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‡Merck & Co., Inc., Rahway, N. J.

TABLE I  
COMPARATIVE STATISTICS ON HUMAN PLAGUE IN SAN FRANCISCO, NEW ORLEANS,  
AND HAMAKUA DISTRICT OF HAWAII

YEAR	CASES	DEATHS	CASE RATE PER 100,000	MORTALITY PERCENTAGE
<i>Hamakua District of Hawaii (1910-1944)</i>				
1910	3	3	30	100
1911	5	5	50	100
1912	7	7	70	100
1913	4	4	40	100
1914	4	4	40	100
1915	4	4	40	100
1916	0	0	0	0
1917	5	0	0	0
1918	0	5	50	100
1919	7	7	70	100
1920	4	4	40	100
1921	4	4	40	100
1922	12	12	120	100
1923	1	1	10	100
1924	2	2	20	100
1925	1	1	10	100
1926	7	7	70	100
1927	7	7	70	100
1928	8	8	80	100
1929	5	4	50	80
1930	0	0	0	0
1931	0	0	0	0
1932	2	2	20	100
1933	2	2	20	100
1934	2	2	20	100
1935	1	1	10	100
1936	0	0	0	0
1937	0	0	0	0
1938	0	0	0	0
1939	1	1	10	100
1940	0	0	0	0
1941	0	0	0	0
1942	0	0	0	0
1943	7	7	70	100
1944	5	5	50	100
Total	111	110	31.7	99.1
<i>San Francisco Outbreak of Plague (Second Outbreak) (May, 1907, to February, 1908)</i>				
1907	158	77	40	49
1908	2	1	0.5	50
Total	160	78	20	49
<i>New Orleans Outbreak of Plague (June, 27, 1914, to Sept. 8, 1915)</i>				
	31	10	8.5	32

Army. The Surgeon General's office stated that at the time penicillin was released for this experiment a similar experiment had been conducted on the mainland on guinea pigs with negative results.

#### EXPERIMENTS

A pure culture of *Pasteurella pestis* organisms was prepared from a rat infected with plague which was found near the town of Honokaa in the north Hamakua area. The organism was identified by morphology, clinical and pathologic reaction in guinea pigs, and by cultural characteristics. Guinea pigs weighing from 250 to 300 grams were used as experimental animals since guinea pigs have a known marked susceptibility to plague. Two thousand five hundred

organisms, as determined by the McFarland nephelometer and Petroff-Hauser counting chamber, were used in each instance to produce plague in the guinea pigs. Inoculations were made intramuscularly or intraperitoneally into animals previously given penicillin or subsequently treated with the drug. When animals died or were sacrificed, organisms were identified as *Pasteurella pestis* by their morphology in tissue smears and by their biochemical reactions in culture media.

*Penicillin Administered Prior to Inoculation with B. pestis* (Table II).—One thousand Florey units of penicillin\* were injected intramuscularly into eight guinea pigs as follows: 72, 48, 24, 8, 4, 3, 2, and 1 hours prior to sub-

TABLE II

PENICILLIN GIVEN PRIOR TO INOCULATION OF GUINEA PIGS WITH 2,500 *B. PESTIS* ORGANISMS

GUINEA PIG	DOSAGE AND ROUTE OF PENICILLIN (UNITS/24 HR.)	TIME FIRST ADMINISTERED (HR. PRIOR)	AMOUNT AND ROUTE OF <i>B. PESTIS</i>	TIME OF DEATH AFTER <i>B. PESTIS</i> INOCULATION (HR.)	TOTAL UNITS PENICILLIN ADMINISTERED	DEATH (HR.) AFTER PENICILLIN FIRST ADMINISTERED	ORGANISM RECOVERED
1	1,000 P	72	2,500 S	96	3,000	168	<i>B. pestis</i>
2	1,000 M	72	2,500 P	100	3,000	172	<i>B. pestis</i>
3	1,000 P	48	2,500 S	140	2,000	188	<i>B. pestis</i>
4	1,000 M	48	2,500 P	244	2,000	292	<i>B. pestis</i>
5	1,000 P	24	2,500 S	48	1,000	72	<i>B. pestis</i>
6	1,000 M	24	2,500 P	244	1,000	268	<i>B. pestis</i>
7	1,000 M	8	2,500 S	60	1,000	68	<i>B. pestis</i>
8	1,000 M	4	2,500 S	68	1,000	64	<i>B. pestis</i>
9	1,000 M	3	2,500 S	72	1,000	75	<i>B. pestis</i>
10	1,000 M	2	2,500 S	96	1,000	98	<i>B. pestis</i>
11	1,000 M	1	2,500 S	70	1,000	71	<i>B. pestis</i>

P, Intraperitoneally.

M, Intramuscularly.

S, Subcutaneously.

cutaneous inoculation with *B. pestis* organisms; the dose was repeated at 24-hour intervals for the two animals started on penicillin 72 and 48 hours before receiving the plague organisms. The same dosage of penicillin was injected intraperitoneally into three animals at 24-hour intervals 72, 48, and 24 hours previous to intraperitoneal inoculation with plague organisms. All of these experimental animals died within 244 hours of the time of their inoculation with plague and at autopsy showed plague organisms in smears and bacteriologic cultures made from liver, spleen, and heart blood.

*Penicillin Administered at the Same Time as Inoculation With B. pestis* (Table III).—Four guinea pigs were given 1,000 units of penicillin, two by intramuscular injection and two by intraperitoneal injection, immediately upon being inoculated with 2,500 *B. pestis* organisms (by the intramuscular route in the case of two animals and by the intraperitoneal route in the other two). The same dosage of penicillin was repeated every 24 hours until death. Four other animals received 300 units of penicillin simultaneously with inoculation with plague organisms and continued to receive 300 units every 3 hours until death. One of these animals was killed after 244 hours. All others died within that period. Cultures and smears at autopsy of these animals showed plague organisms.

\*Dosage was based on report by Hamre, Rake, McKee and MacPhillamy on the toxicity of penicillin for guinea pigs.<sup>3</sup>

TABLE III

PENICILLIN STARTED AT TIME OF INOCULATION OF *B. PESTIS* (2,500) INTO GUINEA PIGS

GUINEA PIG	DOSAGE AND ROUTE OF PENICILLIN	TIME ADMINISTERED	AMOUNT AND ROUTE OF <i>B. PESTIS</i>	TIME OF DEATH AFTER <i>B. PESTIS</i> INOCULATION (HR.)	TOTAL UNITS PENICILLIN ADMINISTERED	DEATH AFTER PENICILLIN FIRST ADMINISTERED (HR.)	ORGANISM RECOVERED
	(UNITS/24 HR.)						
16	1,000 M	Same time as	2,500 P	144	5,000	144	<i>B. pestis</i>
17	1,000 M	Same time as	2,500 S	112	4,000	112	<i>B. pestis</i>
18	1,000 P	Same time as	2,500 P	140	5,000	140	<i>B. pestis</i>
19	1,000 P	Same time as	2,500 S	104	4,000	104	<i>B. pestis</i>
	(UNITS/3 HR.)						
54	300 M	Same time as	2,500 P	144	10,500	144	<i>B. pestis</i>
55	300 M	Same time as	2,500 S	196	14,200	196	<i>B. pestis</i>
56	300 P	Same time as	2,500 P	100	7,500	100	<i>B. pestis</i>
57	300 P	Same time as	2,500 S	Sacrificed after 244	18,400	Sacrificed after 244	<i>B. pestis</i>

P, Intraperitoneally.

M, Intramuscularly.

S, Subcutaneously.

*Penicillin Administered Subsequent to Inoculation With B. pestis* (Table IV).—Penicillin was injected intramuscularly and intraperitoneally in varying dosages into sixty guinea pigs inoculated either subcutaneously or intraperitoneally with 2,500 *B. pestis* organisms. Treatment was begun 24, 48, 72, 96, or 120 hours after inoculation with plague and continued until death or until 244 hours, when the animals were sacrificed and autopsied. In all of these animals *B. pestis* organisms were seen in large numbers in tissue smears at autopsy. Ten thousand units of penicillin were given every 24 hours to four animals with advanced plague beginning 96 hours after plague inoculation in two animals and 120 hours after plague inoculation in the other two animals, and 100,000 units were given each 24 hours to two animals 96 hours after inoculation with plague organisms. Ten thousand units were given every 3 hours to two animals beginning 120 hours after they had received plague organisms. All of these animals receiving heroic dosages died within 120 hours after penicillin administration and were found to have plague organisms as determined by tissue smears and cultures. Larger dosages were not tried in earlier stages of the disease since Hamre and co-workers<sup>2</sup> found that dosages of from 7,000 to 12,000 units of penicillin per kilogram per day were fatal to guinea pigs.

*Controls* (Table V).—Ten animals which were inoculated either subcutaneously or intraperitoneally with 2,500 *B. pestis* organisms received no penicillin. One of these died after 48 hours, one after 72 hours, three after 96 hours, two after 112 hours, one after 216 hours, and two were still alive at 316 hours but were sacrificed at that time. *B. pestis* organisms were present in all of these at death, including the two which were sacrificed. Two guinea pigs given 1,000 units of penicillin per day for eleven doses and two given 300 units every 3 hours

TABLE IV

PENICILLIN ADMINISTERED AFTER INOCULATION OF *B. PESTIS* (2,500) INTO GUINEA PIGS

GUINEA PIG	DOSAGE AND ROUTE OF PENICILLIN	TIME ADMINIS- TERED (HR. AFTER)	AMOUNT AND ROUTE OF <i>B.</i> <i>PESTIS</i>	TIME OF DEATH AFTER <i>B. PESTIS</i> INOCULATION (HR.)	TOTAL UNITS PENI- CILLIN ADMINIS- TERED	DEATH AFTER PENICILLIN FIRST ADMINISTERED (HR.)	ORGAN- ISM RECOV- ERED
	(UNITS/24 HR.)						
20	1,000 M	24	2,500 P	86	2,000	62	<i>B. pestis</i>
21	1,000 M	24	2,500 S	72	1,000	48	<i>B. pestis</i>
22	1,000 P	24	2,500 P	86	2,000	62	<i>B. pestis</i>
23	1,000 P	24	2,500 S	172	5,000	148	<i>B. pestis</i>
24	1,000 M	24	2,500 P	124	3,000	100	<i>B. pestis</i>
25	1,000 M	48	2,500 S	96	1,000	48	<i>B. pestis</i>
26	1,000 P	48	2,500 P	124	3,000	72	<i>B. pestis</i>
27	1,000 P	48	2,500 S	196	6,000	148	<i>B. pestis</i>
28	1,000 M	48	2,500 P	176	5,000	128	<i>B. pestis</i>
29	1,000 M	48	2,500 S	144	3,000	96	<i>B. pestis</i>
30	1,000 P	72	2,500 P	200	5,000	128	<i>B. pestis</i>
31	1,000 P	72	2,500 S	152	3,000	80	<i>B. pestis</i>
32	1,000 M	72	2,500 P	121	2,000	49	<i>B. pestis</i>
33	1,000 M	72	2,500 S	121	2,000	49	<i>B. pestis</i>
34	1,000 P	96	2,500 P	98	1,000	2	<i>B. pestis</i>
35	1,000 P	96	2,500 S	224	5,000	128	<i>B. pestis</i>
36	1,000 M	96	2,500 P	230	5,000	134	<i>B. pestis</i>
37	1,000 M	96	2,500 S	196	4,000	100	<i>B. pestis</i>
38	1,000 P	96	2,500 P	200	4,000	104	<i>B. pestis</i>
39	1,000 P	120	2,500 S	Sacrificed after 244	4,000	Sacrificed after 124	<i>B. pestis</i>
41	1,000 M	96	2,500 S	Sacrificed after 244	5,000	Sacrificed after 148	<i>B. pestis</i>
42	10,000 P	96	2,500 P	148	20,000	52	<i>B. pestis</i>
43	1,000 M	96	2,500 S	200	4,000	104	<i>B. pestis</i>
44	10,000 P	96	2,500 P	196	40,000	100	<i>B. pestis</i>
45	100,000 P	96	2,500 S	172	300,000	76	<i>B. pestis</i>
46	100,000 P	96	2,500 P	172	300,000	76	<i>B. pestis</i>
47	10,000 M	120	2,500 S	240	50,000	120	<i>B. pestis</i>
48	10,000 P	120	2,500 P	240	50,000	120	<i>B. pestis</i>
50	1,000 M	120	2,500 P	216	4,000	96	<i>B. pestis</i>
51	1,000 P	120	2,500 S	172	3,000	50	<i>B. pestis</i>
	(UNITS/3 HR.)						
58	300 M	24	2,500 P	96	5,400	72	<i>B. pestis</i>
59	300 M	24	2,500 S	Sacrificed after 244	16,600	Sacrificed after 220	<i>B. pestis</i>
60	300 P	24	2,500 P	Sacrificed after 244	16,600	Sacrificed after 220	<i>B. pestis</i>
61	300 P	24	2,500 S	220	14,800	196	<i>B. pestis</i>
62	300 M	48	2,500 P	172	9,300	124	<i>B. pestis</i>
63	300 M	48	2,500 S	112	4,800	64	<i>B. pestis</i>
64	300 P	48	2,500 P	72	1,000	24	<i>B. pestis</i>
65	300 P	48	2,500 S	148	9,000	100	<i>B. pestis</i>
66	300 M	72	2,500 P	148	7,200	72	<i>B. pestis</i>
67	300 M	72	2,500 S	172	9,000	100	<i>B. pestis</i>
68	300 P	72	2,500 P	130	6,000	58	<i>B. pestis</i>
69	300 P	72	2,500 S	114	5,100	42	<i>B. pestis</i>
70	300 M	96	2,500 P	148	3,600	52	<i>B. pestis</i>
71	300 M	96	2,500 S	116	3,000	20	<i>B. pestis</i>
72	300 P	96	2,500 P	196	9,000	100	<i>B. pestis</i>
73	300 P	96	2,500 S	196	9,000	100	<i>B. pestis</i>
74	300 M	120	2,500 P	Sacrificed after 244	12,000	Sacrificed after 124	<i>B. pestis</i>
75	300 M	120	2,500 S	172	5,700	52	<i>B. pestis</i>
76	300 P	120	2,500 P	196	7,500	76	<i>B. pestis</i>

P, Intraperitoneally.

M, Intramuscularly.

S, Subcutaneously.



TABLE IV—CONT'D

GUINEA PIG	DOSAGE AND ROUTE OF PENICILLIN	TIME ADMINIS-TERED (HR. AFTER)	AMOUNT AND ROUTE OF B. PESTIS	TIME OF DEATH AFTER B. PESTIS INOCULATION (HR.)	TOTAL UNITS PENI-CILLIN ADMINIS-TERED	DEATH AFTER PENICILLIN FIRST ADMINISTERED (HR.)	ORGAN-ISM RECOV-ERED
	(UNITS/3 HR.)						
77	300 P	120	2,500 S	192	6,900	72	B. pestis
78	5,000 M	120	2,500 P	196	7,500	76	B. pestis
79	5,000 P	120	2,500 S	Sacrificed after 244	205,000	Sacrificed after 124	B. pestis
80	10,000 M	120	2,500 P	148	90,000	28	B. pestis
81	10,000 P	120	2,500 S	172	170,000	52	B. pestis
82	1,000 M	120	2,500 P	172	17,000	52	B. pestis
83	1,000 P	120	2,500 S	196	25,000	76	B. pestis
85	5,000 M	144	2,500 S	196	85,000	52	B. pestis
86	5,000 P	144	2,500 P	196	85,000	52	B. pestis
87	5,000 M	144	2,500 S	196	85,000	52	B. pestis
88	1,000 P	144	2,500 P	Sacrificed after 244	33,000	Sacrificed after 100	B. pestis

P, Intraperitoneally.

M, Intramuscularly.

S, Subcutaneously.

TABLE V

CONTROL GUINEA PIGS INOCULATED WITH B. PESTIS (2,500)

GUINEA PIG	DOSAGE AND ROUTE OF PENICILLIN	TIME ADMINIS-TERED	AMOUNT AND ROUTE OF B. PESTIS	TIME OF DEATH AFTER B. PESTIS INOCULATION (HR.)	TOTAL UNITS PENI-CILLIN ADMINIS-TERED	DEATH AFTER PENICILLIN FIRST ADMINISTERED (HR.)	ORGAN-ISM RECOV-ERED
12	(UNITS DAILY) 1,000 M	72 hr. be-fore	-	Sacrificed after 316	11,000	Sacrificed after 316	None
15	1,000 P	72 hr. be-fore	-	Sacrificed after 316	11,000	Sacrificed after 316	None
52	UNITS/3 HR. 300 M	Same time as	-	Sacrificed after 244	19,200	Sacrificed after 244	None
53	300 P	Same time as	-	Sacrificed after 244	19,200	Sacrificed after 244	None
40	-	-	2,500 P	72	-	-	B. pestis
49	-	-	2,500 S	112	-	-	B. pestis
84	-	-	2,500 P	48	-	-	B. pestis
89	-	-	2,500 S	216	-	-	B. pestis
90	-	-	2,500 P	96	-	-	B. pestis
91	-	-	2,500 S	112	-	-	B. pestis
92	-	-	2,500 P	96	-	-	B. pestis
93	-	-	2,500 S	96	-	-	B. pestis
13	-	-	2,500 P	Alive; sacrificed after 316	-	-	B. pestis
14	-	-	2,500 S	Alive; sacrificed after 316	-	-	B. pestis
98 Normal control	-	-	-	Alive	-	-	-

P, Intraperitoneally.

M, Intramuscularly.

S, Subcutaneously.

until a total of 19,200 units had been given showed no symptoms of illness and no gross or microscopic pathology at autopsy after being sacrificed. Although the widespread variation in the time of death after plague inoculation of the control animals and of the animals receiving penicillin does not permit any accurate statistical comparison, it can be said that there was no apparent prolongation of life caused by penicillin administration.

TABLE VI

IN VITRO EFFECT OF PENICILLIN ON *B. PESTIS* AT 29° C. (2,500,000 *B. PESTIS* ORGANISMS IN 1 C.C. OF PHYSIOLOGIC SALINE SOLUTION)

TOTAL UNITS OF PENICILLIN (IN 10 C.C. DUNHAM'S PEPTONE)	ORGANISM RECOVERED AFTER 6 DAYS
10	<i>B. pestis</i>
100	<i>B. pestis</i>
1,000	<i>B. pestis</i>
10,000	<i>B. pestis</i>
20,000	<i>B. pestis</i>
30,000	<i>B. pestis</i>
40,000	<i>B. pestis</i>
50,000	<i>B. pestis</i>
60,000	<i>B. pestis</i>
70,000	<i>B. pestis</i>
80,000	<i>B. pestis</i>
90,000	<i>B. pestis</i>
100,000	None
200,000	None
300,000	None
400,000	None
500,000	None

*In Vitro* (Table VI).—Sterile test tubes containing 10 c.c. each of Dunham's peptone in which varying concentrations of penicillin had been dissolved were each seeded with 1 c.c. of a saline suspension of a 24-hour veal-infusion agar growth of *Pasteurella pestis*. The *B. pestis* suspension contained approximately 2,500,000 organisms per cubic centimeter. These tubes were incubated at 29° C. for six days and transplants made to test for sterility. Concentrations of penicillin of 100,000 units or greater were required to kill the inoculum of 2,500,000 *B. pestis* organisms.

## CONCLUSIONS

1. Plague is a serious public health problem in the north Hamakua area of the Island of Hawaii.

2. In this experiment penicillin appeared to be of no benefit in combating bubonic plague in guinea pigs.

3. In vitro, a concentration of 100,000 units of penicillin in Dunham's peptone solution was required to kill an inoculum of 2,500,000 *B. pestis* organisms when incubated at 29° C. for six days.

The authors wish to express their appreciation to Sgt. George T. O'Dea and Pfc. John Wilson for their assistance in conducting the in vivo experiments.

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## CONGENITAL HEMOLYTIC ANEMIA IN A NEGRO

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CONGENITAL hemolytic icterus has been reported only once in the Negro race.<sup>1</sup> Because the condition apparently is rare in this race, it seems worthwhile to report the occurrence of such a case which has come under our observation.

### CASE REPORT

A. F., a 14-year-old, dark-skinned Negro girl, was admitted to St. Philips' Hospital April 17, 1944, because of anemia and a low-grade fever of undetermined origin. She had been well until eleven weeks previous to admission. At that time she had developed a sore throat followed by a painful right wrist with no further joint symptoms. At the time of the first examination, her physician found the hemoglobin to be 6.5 Gm. per 100 c.c., and, as she continued to run a low-grade fever, she was referred to the local hospital. There the hemoglobin was recorded as 9.0 Gm., and it was noted that there was enlargement of the heart and spleen. She was given one transfusion and intramuscular liver and bismuth injections, the latter because of the finding of a positive Wassermann. She left the hospital apparently unimproved, and in the interim prior to her admission here, two x-ray pictures of the chest, made because of slight loss of weight, were negative.

Past history was essentially negative.

Family history: The mother died when the patient was 9 days old. No information was available as to the cause of death. The father and one brother are living and in good health. There were no other children.

Physical examination revealed a well-developed, well-nourished Negro girl, with the usual dark color seen in the average Negro in this region, lying in bed in no acute distress; temperature 101° F.; pulse, 134; respirations, 34. There was a slight icteric tint to the sclerae. The heart was moderately enlarged to the left. Roentgenogram of the chest showed a mitral configuration of the heart. There was a loud blowing systolic murmur over the entire precordium, best heard over the pulmonic area. No thrills were noted. Blood pressure was 144/80. The cardiac consultant believed the cardiac findings to be those of chronic anemia. The spleen was markedly enlarged and presented a firm sharp edge. There was slight edema of both ankles. The cervical, axillary, and inguinal nodes were moderately enlarged, discrete, and nontender. All other findings were essentially normal.

Laboratory examination: Electrocardiogram showed sinus tachycardia, a rate of 125 to 136 per minute; no right axis deviation. Urinalysis was normal. Blood examination: red blood cells, 2,520,000 per cubic millimeter; hemoglobin, 6.2 Gm. per 100 c.c.; white blood cells, 15,600, with 82 per cent polymorphonuclear neutrophils, 1 per cent eosinophiles, 1 per cent basophiles, 14 per cent lymphocytes, 2 per cent monocytes; reticulocytes, 17 per cent; icteric index, 16 units. Fragility test using the method of Sanford revealed hemolysis beginning at 0.75 and complete at 0.42; controls, 0.44 to 0.32. The red blood cells did not show the sickling phenomenon. The Wassermann test was negative.

Course: In view of these findings, congenital hemolytic jaundice was thought to be the most plausible diagnosis. The blood film showed typical microspherocytosis with a cell diameter of 7 microns. The fragility test when repeated showed hemolysis starting at 0.70 and completed at 0.42. Biopsy of an axillary lymph node showed moderate hyperplasia with scattered reticulum cells containing blood pigment. With rest in bed, the anemia improved and April 29, the date of splenectomy, the red blood cell count was 2,900,000 per cubic millimeter; the hemoglobin, 8.7 Gm.; and the icterus index, 16 units.

At operation a spleen weighing 465 grams was removed. There was some accessory splenic tissue at the hilus of the organ. The capsule was smooth and glistening and was a

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deep bluish-purple color mottled with several irregular reddish-purple areas. On section the follicles appeared quite prominent, but there appeared to be only a slight increase of fibrous trabeculation. The organ was somewhat firmer than usual. Microscopically, the spleen showed prominent, irregular sinuses and marked congestion of pulp with many cells containing blood pigment. It was thought that the appearance was consistent with the spleen of congenital hemolytic icterus.

By May 14 the patient was greatly improved, and laboratory examination revealed the following: red blood cells, 3,580,000 per cubic millimeter; hemoglobin 10.6 Gm.; white blood cells, 11,150, and a normal differential. Fragility began at 0.60 and was complete at 0.33. This was repeated May 17 when hemolysis began at 0.54 and was complete at 0.39. Microspherocytosis was present and there appeared to be no increased regeneration. The patient was discharged May 20 in excellent condition.

She returned for re-examination Aug. 10, 1944, and appeared to be in perfect health. The cardio-thoracic ratio was found to be reduced to 46 per cent as compared to 55 per cent when the patient was in the hospital before splenectomy. The systolic murmur, believed to be functional, was still present. Laboratory examination showed the following: red blood cells, 4,490,000; hemoglobin, 15.3 Gm.; white blood cells 5,600; and an icterus index of 8 units. Hemolysis began at 0.54 and was complete at 0.30. The blood films showed microspherocytes.

#### FAMILY STUDY

The family was well established in a rural community of Virginia where its members had resided for some years. Questioning of white persons in the community did not shed any light on the possibility of the presence of white blood in the family background. A fourth-year medical student from the com-

TABLE I

RELATIONSHIP	COLOR	MICRO-SPHEROCYTOSIS	MEAN CELL DIAMETER
1. Patient	Dark	+	7.0
2. Aunt on father's side	Tan	-	8.0
3. Grandfather on father's side	Tan	-	7.9
4. Grandmother on father's side	Tan	-	7.7
5. Uncle on mother's side	Tan	-	8.0
6. Uncle on mother's side	Tan	+	6.6
7. Grandmother on mother's side	Dark tan	+	7.2
8. Uncle on father's side	Quite light	-	8.0
9. Aunt on father's side	Typical Negro	-	8.0

munity carried out this survey, securing blood films on the various members of the family. These were studied by Dr. Russell Haden and the results are reported in Table I. Three other members of the family who visited the hospital were also studied, although mean cell diameters were not obtained. In none was the spleen palpable. Studies on these are recorded in Table II.

Blood films obtained from the father and uncle showed small cells, many well filled with hemoglobin and highly suggestive of microspherocytosis. The increased reticulocytes in these two, as shown in Table II, in the light of the high hemoglobin is interesting as is the increased icterus index. Films from the brother showed no microspherocytes, although the cells appeared small for an 18-year-old boy.

TABLE II

	HEMOGLOBIN (PER CENT)	RETICULOCYTES (PER CENT)	ICTERIC INDEX (UNITS)	FRAGILITY
Father	110	3	8	0.42 to 0.30
Uncle on father's side	94	3.2	10.5	0.42 to 0.30
Brother	102	1.2	4.5	0.48 to 0.33

## DISCUSSION

Many textbooks describe congenital hemolytic icterus as occurring "in all races and climates." However, one of us (J. H. S.), in a relatively extensive experience with the blood of Negroes, had never encountered the disease in the race. After ten years' experience as hematologist associated with the large Negro hospital and clinics of the Medical College of Virginia, he had begun to feel that congenital hemolytic icterus did not occur in the Negro, sickle-cell anemia possibly being its counterpart.

Wintrobe<sup>1</sup> states that he has "encountered one instance (of congenital hemolytic icterus) in a Negress of undoubtedly mixed blood." Various reviews<sup>2-6</sup> do not record the disease in the Negro race. Haden<sup>7</sup> and Kracke,<sup>8</sup> both interested in congenital hemolytic icterus, have not observed the disease in a Negro.

The significantly altered erythrocyte diameters in the family group are in the uncle on the mother's side, with a mean cell diameter of 6.6, and the grandfather on the mother's side. The uncle was described by the investigator as "tan," the grandfather as "dark tan."

A confusing finding is the definite microspherocytic tendency with increased reticulocytes and icteric index on the father's side. It would be assuming too much to suggest the presence of the trait on both sides of the family. The father could recall no history of jaundice in the family.

It is possible that congenital hemolytic anemia does occur in full-blooded Negroes, although it has not been recorded previously. *Infectious mononucleosis*, for instance, only recently has been observed in the race. It may well be that less adequate hematologic studies have contributed to this oversight. On the other hand, the interbreeding of the trait through miscegenation in the family background cannot be excluded. The disease in the Negro must be rare, and this rarely is to be expected if the trait is found in the Negro only after inbreeding. Congenital hemolytic anemia is a comparatively uncommon disease in the white race. The probability of transmission through miscegenation must be infinitesimal.

## SUMMARY

The first case of congenital hemolytic anemia to be reported in detail in the Negro race is recorded. Apparently the disease, though rare, does occur among Negroes. The probability of transmission of the trait through miscegenation is discussed.

The authors wish to acknowledge the kindness of Dr. Russell L. Haden, for measuring the red cell diameters, and of Mr. E. G. Field, for conducting the family survey.

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## THE LIFE DURATION OF THE RED BLOOD CELL OF THE MACACUS RHEBUS MONKEY

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MANY efforts have been made to establish the life duration of the erythrocyte in the circulating blood. Such diverse mammalian forms as the rat, rabbit, dog, monkey, and man have been studied and many different approaches to the problem have been used. It is not the purpose of this paper to cover the literature on the subject more than to indicate the chief avenues of attack.

Ashby<sup>1</sup> employed differential agglutination of transfused blood cells as an index of their persistence and concluded that the erythrocyte of man possessed a life duration of thirty days or more. Later, Isaacs<sup>2</sup> showed that the agglutination response could not be relied upon to identify transfused red cells beyond the second to the fourth day following transfusion. Eaton and Damren<sup>3</sup> utilized the young erythrocyte (or reticulocyte) as an indicator of red cell replacement following hemorrhage. They showed that in the rabbit following hemorrhage new groups of reticulocytes invade the circulation rhythmically at intervals of from eight to nine days. Such intervals were interpreted as representing the life duration of the red blood cell in this animal. Escobar and Baldwin<sup>4</sup> studied the effects of altered oxygen tension on the blood of rats. The time required for restoring the erythrocyte count to normal following lowered oxygen tension was interpreted as representing the life duration of the circulating red blood cell. This they found to be from eleven to thirteen days. Hawkins and Whipple<sup>5</sup> employed bile pigment output as an indicator of erythrocytic disintegration. These workers found the interval between a sudden blood depletion in a dog and a subsequent sudden rise in bile pigment output to average 124 days. This time interval, they believe, represents the days between the influx of an erythrocyte population induced by sudden blood cell depletion and the subsequent time of disintegration of this cell group and hence represents the life of the red blood cell of the dog. Harne and associates<sup>6</sup> using the approach of Eaton and Damren, studied the blood of the albino rat. It was found that the life span of the red blood cell was between eight and nine days. This life span agrees with that of the rabbit.

In the present study monkeys were subjected to acute hemorrhage and the changes in the blood picture which follow were observed daily. Not all these observations will be discussed here. In this paper we shall consider the experimental procedure and those results which relate specifically to the life span of the red cell.

It has been established in all animal forms thus far studied that when large numbers of red cells are removed from an animal's circulation by hemorrhage, the cells which are thus lost will be promptly replaced by a large number of

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reticulocytes. When supravitality stained, these reticulocytes can be identified and counted. Thus by using the reticulocyte count as an indicator, it is possible to evaluate the degree of red cell replacement.

It is obvious that when a population of circulating red blood cells is abruptly removed, as by hemorrhage, and a group of young cells promptly replaces those lost, there will exist in the blood stream a group of red cells all of which are approximately the same age. If all red cells have about the same span of life, a block of such young cells should mature together and go on through life as a functioning unit, finally to become senile and disappear en masse. This physiologic depletion of a cell population simulates in effect a depletion by hemorrhage and thus spontaneously initiates a replacement reticulocyte shower. This spontaneous replacement of cells can again be detected and evaluated as before by the reticulocyte count. Such a sequence of reactions has been shown to occur in all animal forms thus far tested.<sup>3, 6</sup>

The time lapse between the appearance of the first replacement block of young red cells following hemorrhage and that of the second (spontaneous) block should express the life tenure of the red blood cell in the animal studied. This paper presents the evidence of such a sequence as it occurs in the monkey.

#### ANIMAL MATERIAL

Ten young macacus rhesus monkeys were used, four males and six females. The ages at the beginning of the experiment (September, 1941) were estimated to be between 6 and 7 years (Table I). They were kept in separate cages except when permitted to exercise together. Each animal was fitted with a stout collar and a chain by which it could be controlled. For a period of several months preceding experimentation all were schooled to leave the cage and climb upon a table located near by. Here they were covered with a net which was secured

TABLE I  
SHOWING AGE, SEX, WEIGHT, AND CONDITION OF ANIMAL MATERIAL WITH NOTES ON  
TRAINING AND INDIVIDUALITY

ANIMAL NO.	SEX	ESTI-MATED AGE (YR.)	WEIGHT (KG.)	PHYSICAL CONDI-TION	REACTION TO TRAINING	NOTATIONS ON INDIVIDUALITY
1	F	7	4.6	Good	Poor	Never became tame; would nervously assist operator with restraining net; excellent subject
2	F	7	3.6	Good	Fair	Ugly, mean, and thievish
3	F	7	4.	Good	Excellent	Good subject; bossy; amorous
4	F	7	4.	Good	Excellent	Good subject; shakes hands timidly; shy
5	M	7	4.	Good	Excellent	Good subject; would be boss of colony
6	M	7	4.4	Good	Excellent	Good subject; vain; thierish; very playful
7	M	6	4.4	Good	Good	Good subject; big bluffers; playful
8	M	6	4.	Good	Poor	Does only what he has to
9	F	6	3.6	Good	Fair	Responsive; devilish
10	F	6	3.4	Good	Fair	Responsive but does things her own way

to the table top by means of a system of sliding blocks. Under the net the animals were petted and offered favorite articles of food.

Eventually they would accept the food and sit quietly while being handled. Each responded in a different way to the total treatment, but all learned to climb upon the table and wait to be covered with the net. One animal (No. 1) actually learned to assist the technician with the preparations (Table I). At no time during the experiment did we notice any evidence of resentment or objection to the tests.

*The net* used was an ordinary pickarel net 18 inches deep, mounted upon a square frame made from  $\frac{3}{8}$  inch metal rod bent into a 14-inch square. The ends of the rod were bent out and brazed together to form a handle.

*Taking the Blood.*—Blood for the various tests was obtained by three different methods. Small amounts of blood for the hemoglobin estimation and reticulocyte, differential, red and white cell counts were taken from the ear

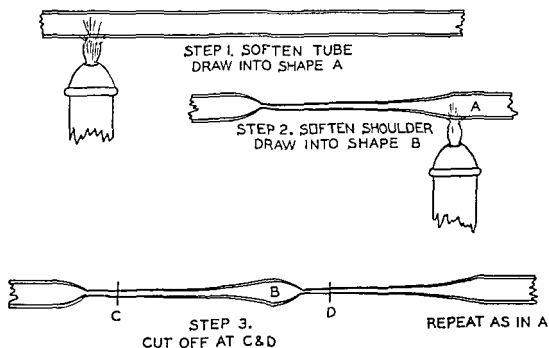


Fig. 1.—Showing steps in the fabrication of the blood-collecting and staining pipette.

by quickly snipping the free auricular border with a pair of small sharp scissors. Only the reticulocyte preparations so obtained are further considered in this paper. Larger amounts of blood, 1 to 5 c.c. for hematocrit determinations, were taken from the superficial veins of the legs, and the largest amounts, 5 to 50 c.c., the so-called hemorrhage, used to induce reticulocytosis, were taken from the femoral vein after the latter had been exposed under light anesthesia.\*

*Preparation of Reticulocyte Films.*—The best preparations of reticulocytes in monkey blood were obtained by the pipette method. This requires a blood pipette made for the purpose and a proper staining solution.

*Pipette.*—The pipette used (Fig. 1, B) is spindle shaped and about 12 cm. long. The caliber at the tip is about 1.5 mm. and at the expanded central portion, about 6 to 8 mm. The distal end is not important except that it must be open. The pipette can be drawn into shape from a piece of ordinary glass tubing 15 mm. in diameter. In fabricating it, the end of the tube is first drawn

\*Anesthesia was induced by the intravenous injection of a fresh aqueous solution of 4 per cent Nembutal— $\frac{3}{4}$  c.c. per kilogram body weight.



out to the length and diameter required to form the tip of the pipette. The flame of the blast lamp is then played upon the shoulder of the drawn out portion (Fig. 1, *A*) to soften it just before it is pulled into shape *B*, Fig. 1. This method gives a good taper to the spindle and a sufficiently large bulb to insure proper functioning.

*The Staining Solution.*—The stain is prepared as follows:

Brilliant cresyl blue	1 per cent
Potassium oxalate	.5 per cent
Merthiolate (1-1,000)	.025 per cent
Sodium chloride	.8 per cent

Dissolve the brilliant cresyl blue in distilled water at room temperature by shaking for fifteen minutes in a shaker. Let stand overnight. Shake again for fifteen minutes and filter through a medium textured paper which is rapid and fairly retentive. Add the oxalate, merthiolate, and sodium chloride. Make up to volume with distilled water and the stain is ready for use. The solution keeps very well but must be stored in a tightly stoppered bottle.

*Use of the Pipette.*—Hold the pipette in a *vertical* position; touch its tip to the surface of the staining solution. Capillary attraction will cause the stain to rise in the pipette to a distance of from 1.5 to 2 cm., depending upon the caliber of the capillary. The stain will remain in position until used unless it is handled roughly or touched against an absorbent. The stain should not be kept in the pipette too long or evaporation will change its ion concentration and so affect its isotonicity with blood.

Hold the pipette in a horizontal position and touch the tip, filled to the end with stain, to the surface of the freshly formed drop of blood. Blood at once enters the pipette. The amount of blood so admitted is not too important, but it is well to limit the quantity to an amount equal to the volume of stain used. *Mix* the stain and blood promptly by first holding the tip of the pipette up until the mixture flows down almost to the bulb and then quickly turning the tip down. Do this several times, or until mixing is complete. Do not hold the pipette tip up too long or the blood will flow into the opposite end of the pipette, air-locking the contents. This does not harm the preparation but makes it very difficult to agitate and remove the mixture. *Smears* should be made from the pipette after about five minutes. First agitate the contents as described, and then immediately place a small drop of the mixture upon the surface of a *perfectly clean* slide and spread it in the usual manner, using the end of another clean slide as a spreader. Allow the films to dry in the air; mount in balsam if permanent preparations are desired. *Examination* of the films may be made immediately without counterstaining, but some prefer to counterstain with Wright's blood stain. Whether counterstained or not, these preparations hold up very well.

Another type of preparation is now being used extensively and can be made from the blood-stain mixture in the pipette as follows: Place a medium sized drop of the blood-stain mixture in the center of a *clean slide* and cover it with a *clean* cover slip. Seal the edges of the cover slip with *paraffin*. Allow to stand for a few minutes to permit the cells to settle and examine as usual.

This so-called "wet-preparation" is by far the best method for studying the cells and their inclusions. The preparations so made, however, are *not* permanent.

*Counting the Reticulocytes.*—The reticulocyte count is accomplished by first exploring the smear under low power for an area in which the cells are evenly distributed and moderately separated. The area thus located is then studied under oil immersion and usually found to contain about 100 red cells per field. Starting from a given point within this area, and counting adjacent fields, 1,000 red cells are counted. The reticulocytes are recorded separately for each block of 100 red cells. This block system of recording is employed to give data on the distribution of the reticulocytes within the area counted. Upon completion of the count the reticulocytes are summed up and expressed in per cent or, better still, as reticulocytes per 1000 red cells.

*Reticulocyte Level in the Monkey Under Control Conditions.*—Tests were begun as soon as the animals became accustomed to the training routine. Each animal underwent standardization of his own reticulocyte output by being subjected to daily reticulocyte counts. Experience showed that from ten to twenty days were sufficient to get the pattern of reticulocyte production (Table II).

Two animals (Nos. 2 and 6, Fig 2) were kept as controls throughout the course of the experiments. Both were ultimately subjected to acute hemorrhage and reacted as normal animals to this procedure. The terminal record of Animal No. 2 appears as No. 2, Series 2, Fig. 4. The terminal record of Animal No. 6 does not appear, as the experiment was terminated after the initial reaction. In this way, all animals of the colony contributed to the results reported here. The combined control and experimental periods of Animals 2 and 6 afforded continuous daily records of the reticulocyte counts for 287 and 220 days, respectively.

The average control level of reticulocytes for the colony (ten animals) was 10.9 reticulocytes per 1,000 red cells. Coincidentally the average reticulocyte level of the male control animals (No. 6), whose control period extended over a period of 174 days, was also 10.9.

The average control count for the males (four animals) was 10.3 and for the females (six animals), 11.3 per 1,000 red cells.

The highest average level in a single animal was 16.7 per 1,000 over a twenty-day period, found in a female (No. 1, Series 2, Table II) also (No. 1, Series 2, Fig. 4). The lowest record, 8.5 per 1,000 over a 70-day period, was shared by two males (Nos. 7 and 8, Table II, and Nos. 7 and 8, Fig. 3). The initial high level of Animal 1 was accepted as a control value because of its constancy even though the value seemed high. Subsequent analyses of these data show that this was not an error.

It is seen (Fig. 2) that the average value of 10 reticulocytes per 1,000 is about correct for the monkey when determinations are carried out over long periods, as this line cuts the long control curves of Animals Nos. 2 and 6 through the center. There are long periods, however, when the curves rise above or fall below this level. We believe, therefore, that the variation in the control levels of the experimental animals which showed variations can be ascribed to the varying activity of cell production at the time the control tests were made

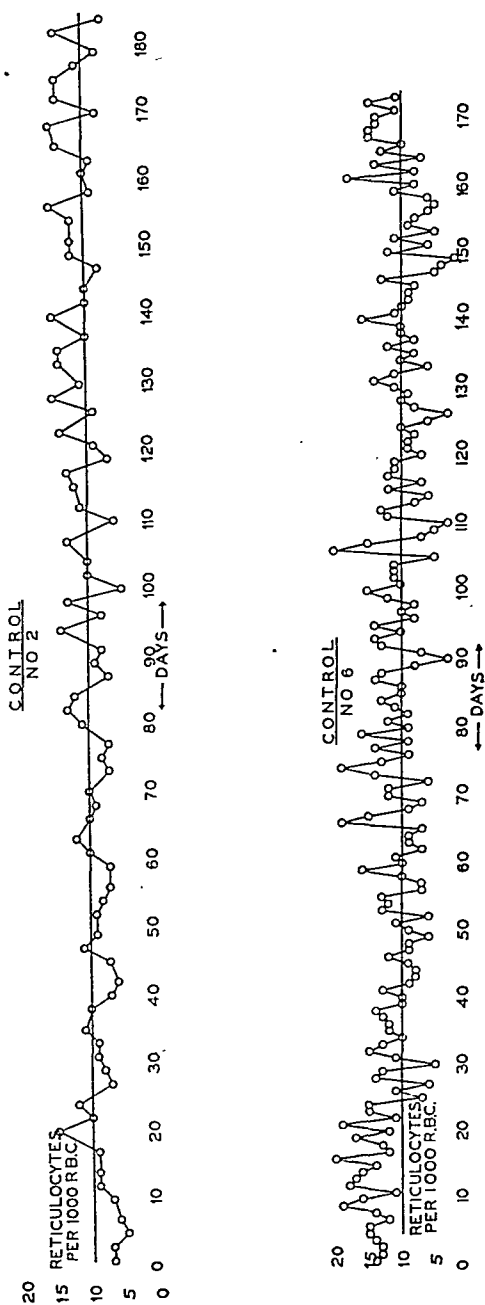


Fig. 2.—Showing control reticulocyte counts in Animals Nos. 2 and 6 over periods of 185 and 174 days, respectively.

TABLE II

SHOWING (1) LENGTH OF THE CONTROL PERIOD, (2) TYPE AND AMOUNT OF HEMORRHAGE, (3) CONTROL RETICULOCYTE LEVEL AS FOUND BY DAILY COUNTS, AND (4) LENGTH OF LIFE OF RED BLOOD CELL

ANIMAL NO. AND EXPERIMENT NO.	NAME AND SEX	CONTROL PERIOD (DAYS)	TYPE OF HEMORRHAGE AND AMOUNT	AVERAGE CONTROL LEVEL RETICULOCYTES (PER 1,000 R.B.C.)	LENGTH OF LIFE RED BLOOD CELL (DAYS)
1	Jiggs F	20	Acute 40 c.c.	8.8	105
1, Series 2	Jiggs F	20	Fractional 9.9-9.9 c.c.	16.7	103
2	Susie F	185	Control	10.5	Control
2, Series	Susie F	10	Fractional 10-10-10-10 c.c.	16.1	112
3	Oscar F	20	Acute 41 c.c.	9.2	94
4	Windy F	20	Fractional 12.5-19-15-16 c.c.	11.2	101
5	Spike M	20	Fractional 23-23-23-23 c.c.	14.3	102 <sub>1</sub>
5, Series	Spike M	20	Acute 43 c.c.	10.	103
6	Pete M	174	Control	10.9	Control
7	Bufo M	70	Acute 40 c.c.	8.5	111 Estimated
8	Lightning M	70	Acute 43 c.c.	8.5	117
9	Flo F	81	Acute 27 c.c.	11.2	
10	Tess F	81	Acute 34 c.c.	12.2	

and is to be expected when *short* control periods are used. Animal No. 1 was a good example. This animal gave widely different control values at periods separated by intervals of six months (16.7 and 8.8, respectively), using twenty days in each case as the control period.

*The Effect of Hemorrhage Upon the Hemopoietic System.*—Following the establishment of individual control reticulocyte values, eight of the ten animals were bled. The amount and type of hemorrhage so induced are shown in Table II and in Figs. 3 and 4. Subsequent to bleeding, daily reticulocyte counts were continued for a period of approximately 130 days and the data so obtained plotted. The resulting curve resolves itself into three parts. The first or initial phase extends over a period of from twenty to thirty days. During this phase the reticulocytes rapidly increase in number and then subside to control level or lower. In the second or interval period the reticulocyte count remains at approximately control level. This extends to about the eightieth day, following which a recrudescence of reticulocytes produces a second elevation or spontaneous phase. The latter and its return to control level constitute the terminal phase of the curve. The first elevation is interpreted as a mass influx of red cells from the bone marrow to replace the loss occasioned by bleeding. The second period is regarded as a stabilized phase following the

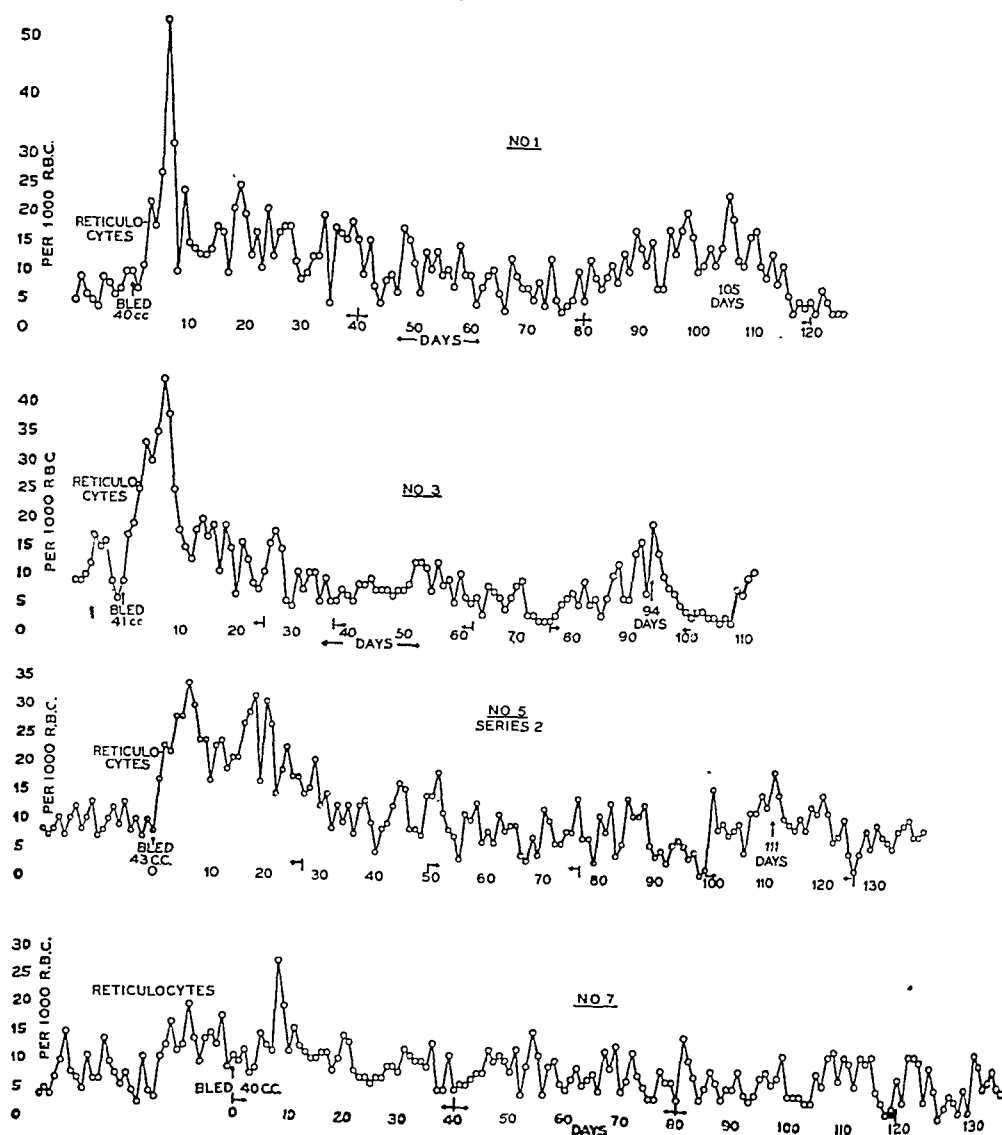


Fig. 3.—Showing reticulocyte counts following the withdrawal of blood equivalent to 1 per cent of body weight at one operation.

restoration of the blood population to normal. The third period represents a spontaneous reticulocytosis, the function of which is to replace the group of cells released during the first period following the hemorrhage and which are now disappearing more or less in mass from the circulation.

This three-phase type of curve has been obtained in eight of eleven experiments (Table III). In seven experiments, the animals were deprived of blood equivalent to 1 per cent of their body weight by a single operation (Fig. 3). In four experiments the animals were bled fractionally; approximately .5 per cent of the body weight in blood was removed at intervals of seventy-two hours (Fig. 4 and Table II). All animals (four) bled fractionally gave the three-

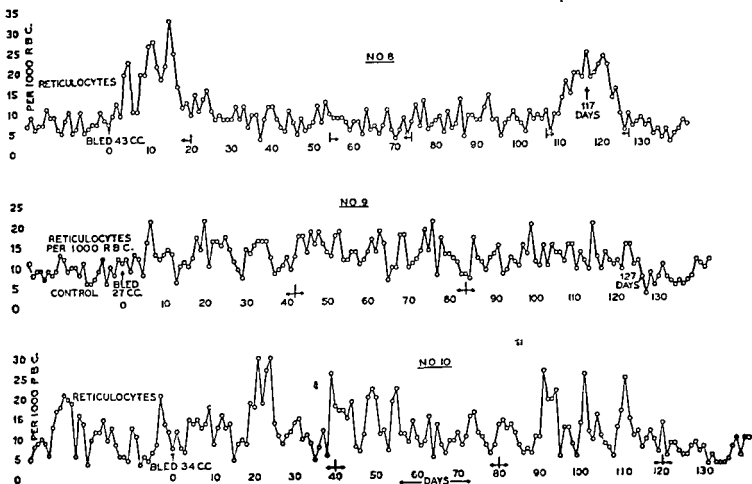


Fig. 3.—(Cont'd)

phase type of curve described. It should be pointed out, however, that the interval period in each of these experiments shows qualitative deviations from the classical picture and are not as smooth as the more typical curves in Fig. 3, which were obtained by single acute withdrawals of blood.

**Discussion.**—From the data submitted it is seen that animals in eight of eleven experiments unquestionably exhibit a spontaneous reticulocytosis after recovery from acute hemorrhage (Table III). This spontaneous reaction always appears after the eleventh week following hemorrhage and in no case has it been seen during the interim. That this is a specific reaction and not a coincidence is proved by the fact that it is completely lacking in the control animals (Fig. 2).

Due to the normal daily fluctuation of the reticulocyte counts in some subjects, it is not always easy to recognize a spontaneous reticulocytosis when it is actually present. (No. 5, Fig. 3). Indeed, without the evidence provided by more pronounced reactions, it is doubtful if such lesser reactions would be recognized at all. However, if, instead of stressing the reticulocyte peak reached in any one day, the volume of red cells being thrown into the circulation over a

TABLE III  
SHOWING NUMBER OF EXPERIMENTS AND TYPES OF REACTIONS

Number of experiments reported	13
Number of controls	2
Reaction to hemorrhage	
Number of animals which did not respond typically	3
Number of animals giving spike reaction	3
Number of animals giving sustained reaction	5
Number of spontaneous reactions	8

period of days is computed (No. 1, Fig. 4 and Table IV), such mild reactions as that shown by No. 5, Fig. 3, assumes its proper significance. When this is done it is seen that the *first phase* of the reticulocyte curve always shows a maximum volume of reticulocytes; the *interval period*, a minimum reticulocyte value; and the *terminal or spontaneous reaction*, a level somewhere between

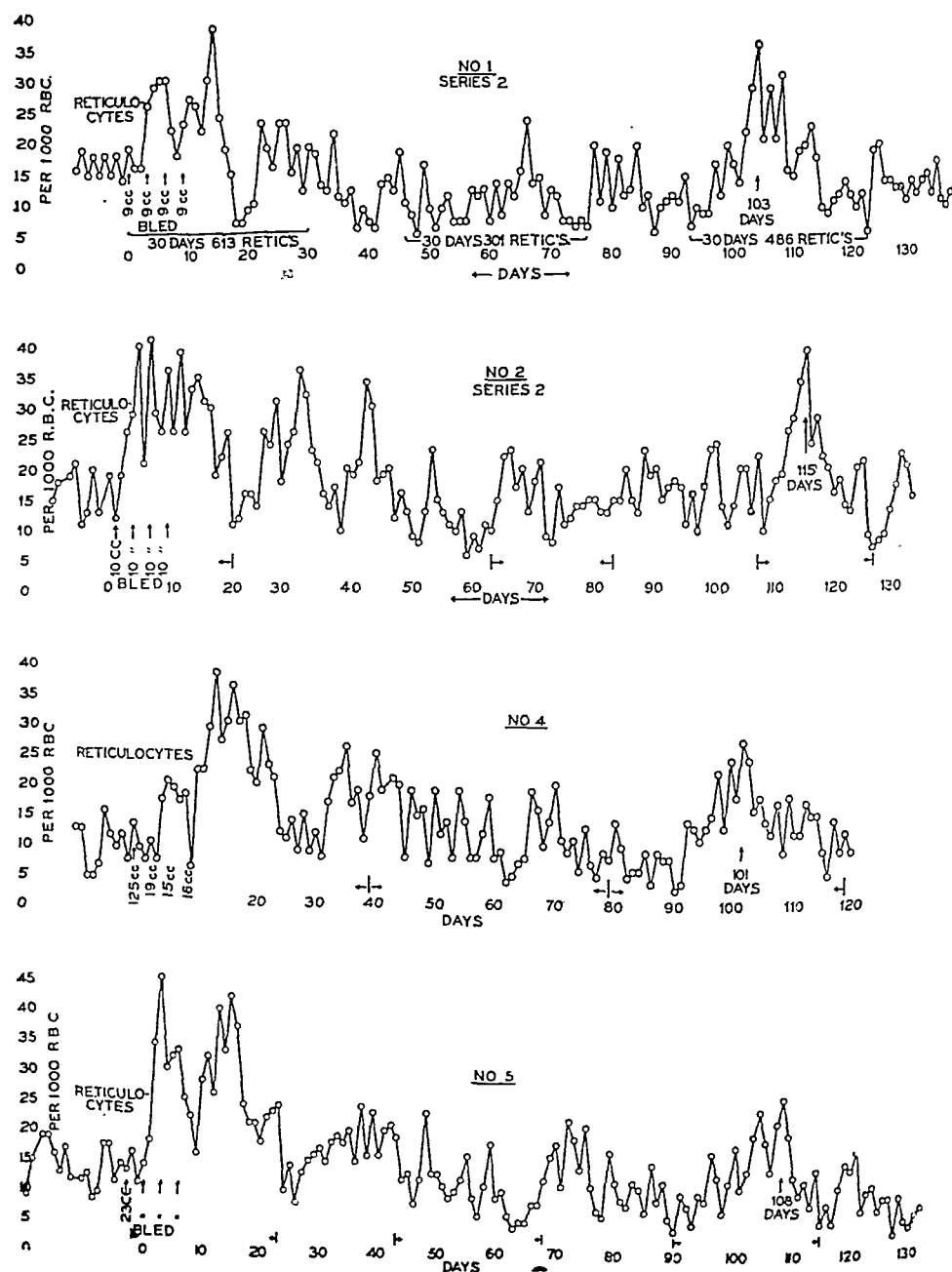


Fig. 4.—Showing reticulocyte counts following four fractional bleedings, each equivalent to approximately .5 per cent of the body weight in blood at intervals of seventy-two hours.

TABLE IV

SHOWING RETICULOCTE OUTPUT OF EACH ANIMAL (EXCEPT CONTROLS) DURING (1) CONTROL PERIOD, (2) INITIAL REACTION, (3) INTERVAL, AND (4) SPONTANEOUS REACTION\*

ANIMAL NO. AND EXPERIMENT NO.	REACTION TYPE	NUMBER OF DAYS UTILIZED IN THE SUBSEQUENT FOUR COMPUTATIONS	RETICULOCTE OUTPUT DURING PERIOD OF DAYS CITED IN COL. 3							
			OUTPUT AT CONTROL LEVEL OR 100 PER CENT (CELLS)	INITIAL REACTION		INTERVAL OUTPUT		SPONTANEOUS REACTION		
				OUTPUT (CELLS)	PER CENT OF NORMAL	OUTPUT (CELLS)	PER CENT OF NORMAL	OUTPUT (CELLS)	PER CENT OF NORMAL	
1	Spike	40	372	681	183	336	98.5	506	136	
1, Series 2	Sustained	30	279	613	220	301	108	456	173	
2	Control	183	Control	Control	Control	Control	Control	Control	Control	
2, Series 2	Sustained	20	178	373	321	287	135	409	229	
3	Spike	25	265	490	185	219	71	224	84	
4	Sustained	40	400	809	201	509	127	560	140	
5	Sustained	25	250	696	278	288	115	362	145	
5, Series 2	Sustained	27	270	624	231	261	93	315	117	
6	Control	174	Control	Control	Control	Control	Control	Control	Control	
7	Spike	40	332	453	136	360	108	343	103	
8	Sustained	20	166	376	226	127	76.5	250	169	
9	Sustained	42	420	523	125	556	132	461	110	
10	Sustained	40	488	548	112	456	100	469	96	

\*The control output in each case is considered 100 per cent (column 4). The second column in each of the last three divisions shows the percentage deviation from control level. Column 3 shows the number of days covered in each of the four periods evaluated and (except the control periods) are indicated by characters (—) beneath each curve. The duration of the spontaneous reaction in days was generally the period used in column 3 (seven instances), but in four cases the curves were divided into thirds. The reticulocyte output in columns 4, 5, 7, and 9 represents the sums of the daily values expressed as reticulocytes per 1,000 red cells for the period of days shown in column 3.

those of the first and second periods (Table IV). In some cases the spontaneous reaction, as recorded in Table IV, does not seem to have occurred at all, as, for example, in Animal No. 3. Here the percentage of reticulocytes at the time of the spontaneous reaction is only 84 per cent of control level for this particular animal. If, however, the plotted curve of this animal is studied, it is clearly evident that the usual spontaneous reaction has occurred (No. 3, Fig. 3). The ratios of the three phases of the cycle are also maintained.

Not all animals of this series responded in the usual manner to the loss of blood (Table III). Three (Nos. 7, 9, and 10, Fig. 3) gave a different pattern of reaction. All were bled the equivalent of 1 per cent of their body weight in blood at one operation. All gave similar over-all pictures of reticulocyte production in that the output in each case was high in the initial phase (this was typical), remained high during the interval and terminal phases, and tapered off finally (after about 120 days) to approximately control level in cadence with the activity of other animals which reacted typically. Whether the type of hemorrhage used to induce the reaction is responsible for these atypical responses, we are unable to say. Work is in progress to determine the cause.

*Life Duration of the Red Blood Cell as Indicated by the Occurrence of Spontaneous Reticulocyte Showers Following Hemorrhage.*—Spontaneous reticulocyte showers whose peaks occur within the terminal phases of the reticulocyte curves, 94 to 117 days following hemorrhage, are interpreted as physiologic reactions to the rapid depletions of the red cell populations resulting from



the disappearance (due to age) of the blocks of cells which were suddenly released by the bone marrow in the initial phase of the experiments to replace those removed by hemorrhage.

Some of these replacement cells begin to disappear about eighty days after the induction of the hemorrhage (or after their appearance). The greatest number, however, disappears from ten to twenty-five days later, while a few persist for as long as forty days. This means that there is a spread in the life span of the red cell of from 80 to 120 days, which figures can be derived from the curves in Figs. 3 and 4 by counting the days between the initial and spontaneous reactions.

The values recorded upon the curves of Figs. 3 and 4 at the peak of each spontaneous reaction represent the time lapse between the induction of the hemorrhage and the highest point of the terminal or spontaneous reaction. The figures obtained by this type of experimentation would place the *average life duration* of the red blood cell of the macacus rhesus monkey at about 100 days.

#### SUMMARY

1. A method is given for making permanent preparations of reticulocytes in monkey blood.

2. Curves are presented showing daily reticulocyte counts in two control and eight experimental animals covering periods of from 120 to 170 days.

3. Two types of blood depletion were employed to stimulate hemopoiesis: (1) withdrawal of large amounts (1 per cent of the body weight) at one operation and (2) fractional withdrawal of .5 per cent of the body weight in blood, at intervals of 72 hours (usually four operations).

4. The span of life of the red blood cell of the macacus rhesus monkey was determined by autonomous blood replacement following hemorrhage. When this method is employed, a spontaneous reticulocyte shower occurs from 94 to 117 days later, indicating a reaction to the mass disappearance of the block of red cells originally mobilized to replace those lost by hemorrhage. The time lapse between the induction of the hemorrhage and the peak of the spontaneous reaction is interpreted to be the life span of the red blood cell in this animal. We realize that from these figures must be deducted the latent period of reaction, which in the monkey was found to be from two to four days.

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## OXYURIASIS

### SIMPLIFIED METHOD OF DIAGNOSIS WITH GLASS SLIDE; INCIDENCE IN A MINNESOTA STATE HOSPITAL; RESULT OF TREATMENT WITH GENTIAN VIOLET

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AT THE Willmar (Minnesota) State Hospital it was noted that differential blood counts of patients frequently showed 4 per cent or more eosinophiles. Since routine search for intestinal parasites in such cases usually revealed pin-worm infection, we decided to make a complete survey of the entire institution. This was commenced in September, 1940, and completed in July, 1942.

The institution, which is built on the cottage plan, has a capacity of 1,450 beds. Each cottage accommodates about 100 patients. Chronic mental patients, transferred from other hospitals, constitute the great majority of the population. Most of them have been hospitalized for more than a year and many for more than a decade. Although this group is fairly stationary, there are frequent transfers of patients from one cottage to another. Alcoholic patients and drug addicts form the minority. They are admitted directly and, as a rule, remain only from two to four months. Except for those in the hospital at the beginning of the survey, the patients were examined on admission. This group, numbering from forty-five to eighty, was housed largely in a separate ward.

#### METHOD OF DIAGNOSIS

At first we used the so-called NIH cellophane swab described by Hall.<sup>1</sup> As this was rather cumbersome for survey work, we began to use a strip of cellophane folded over a tongue blade. At the time we did not find mention in the literature of this method. Since this time, however, a somewhat similar technique has been described by Jacobs.<sup>2</sup> The cellophane tape proved as efficient as the NIH swab but still too slow for our purpose. We then commenced to use glass slides with smooth edges of the type ordinarily employed in microscopic work. These were found not only as accurate as the NIH swab and the cellophane tape, but much faster.

A number was etched or scratched on one end of the slide. This served as an identification not only of the slide, but of the exposed side as well. Metal slide holders were made with spacings far enough apart so that the slides could be handled easily without one touching the other. Metal was used for holders in preference to wood because it was easier to clean. The glass slides were cleaned and used repeatedly.

The smears were made sometime between midnight and 4 A.M. No patient to be examined was allowed to take a bath the night before. The patient was asked to bend over and strain slightly. The examiner would hold the marked end of the slide and press the other end against the anal mucosa and mucocutaneous junction in such a manner that one edge of the slide would be toward

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the center of the anus. The slide would then be lifted and the opposite edge pressed against the opposite anal margin. Care was taken to hold the slide in such a manner that only the marked side would be exposed.

It was found advantageous not to have any fecal matter stick to the slide. The moisture of the anal mucosa was sufficient to make the ova adhere to the glass. It was seldom necessary to examine the entire width of the slide. The ova as a rule were found along the edges. If necessary, the slides could be kept for several days before the microscopic examination was made. By the use of low power and a wide eyepiece, a slide could be examined in a few-moments, especially when ova were present.

All patients in a cottage were examined the same night. In cases in which no ova were found, the examination was repeated at a later date until the slides from all remaining patients were negative. In some cases as many as eighteen examinations were made. Ova were found fairly frequently in large numbers on slides from patients who repeatedly had been found free from ova.

#### INCIDENCE

The results for each cottage are given in Table I. Of the 1,871 persons examined, 1,100 (59 per cent) were found to be infected with pinworms. The incidence of the infection was 72 per cent among the chronic mental patients as compared with 1 per cent among the inebriates and none among the employees. Two of the employees, however, had previously been treated for oxyuriasis.

TABLE I

INCIDENCE OF PINWORM INFECTION AND RESULT OF ONE COURSE OF TREATMENT  
WITH GENTIAN VIOLET

COTTAGE OR GROUP	SEX OF PERSONS EXAMINED	PERSONS EXAMINED	INFECTED BEFORE TREATMENT		INFECTED AFTER ONE COURSE OF TREATMENT	
			NUMBER	PER CENT*	NUMBER	PER CENT†
3	F	83	9	11	0	0
4	M	103	91	88	16	18
5	M	100	40	40	2	5
6	M	106	101	95	6	6
7	F	98	35	36	3	9
8	F	98	69	70	5	7
9	F	84	75	89	7	9
10	F	96	71	74	14	20
11	F	104	94	90	9	10
12	M	102	98	96	1	1
13	M	101	97	96	10	10
14	F	103	89	86	17	19
15	M	99	78	79	3	4
16	M	106	101	95	5	5
Transfers	M	90	38	42	0	0
Transfers	F	53	11	21	0	0
Inebriates	F and M	315	3	1	0	0
Employees	F and M	30	0	0	0	0
Total	F and M	1,871	1,100	59	98	9

\*Of persons examined.

†Of persons infected before treatment.

The incidence of oxyuriasis was decidedly higher in the male than in the female population. It was highest in the disturbed and the deteriorated groups. That the condition is not peculiar to the Willmar State Hospital is evidenced by

the incidence among patients received by transfer during the time of the survey. The lower incidence among these is probably due to shorter periods of hospitalization.

#### TREATMENT

All patients found to be infected were treated with gentian violet. Two  $\frac{1}{2}$ -grain (0.032 Gm.) enteric-coated tablets were given by mouth twice a day after meals for eight days. After a rest period of eight days, the course was repeated. A third course was given after another eight-day rest period. Thus, 48 grains (3.1 Gm.) of the drug were administered over a period of forty days.

Toxic symptoms due to medication were encountered rarely among the male but fairly frequently among the female patients. These symptoms were anorexia, nausea, vomiting, diarrhea, and mild abdominal pain. Some patients lost weight during the period of treatment. The symptoms disappeared when the medication was suspended for a few days. The therapy was then resumed: either the before-mentioned plan was continued or  $\frac{1}{2}$  grain (0.032 Gm.) of the drug was given twice a day continuously for thirty-five days.

After completion of the treatment, search was again made for ova. Six examinations were made in the course of four weeks. Ova were found in ninety-eight (9 per cent of those infected before treatment) after the first course, eleven (1 per cent) after the second, and two (0.2 per cent) after the third. One patient remained infected after five courses of treatment.

The patients in cottages 6 and 12 were re-examined ten months after treatment. Thirty-one (29 per cent) of the former and forty-six (45 per cent) of the latter were found to be infected. Circumstances did not allow us to continue the study. A total of 18,446 slides were examined.

#### SUMMARY

A simplified method of diagnosing pinworm infection is described. Of 1,871 persons examined in a Minnesota state hospital, 1,100 were infected with pinworms. The incidence of infection was 72 per cent among the chronic mental patients, 1 per cent among the inebriates, and none among the employees. One course of treatment with gentian violet was effective in 91 per cent of cases. Of 208 patients examined ten months after treatment, seventy-seven (37 per cent) were found to be infected.

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# A CASE OF TRICHOSTRONGYLUS INFECTION WITH NOTES ON THE IDENTIFICATION OF OVA

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THOUGH various species of *Trichostrongylus* are frequently found in the intestines of ruminants, instances of human infection occurring in the United States are exceedingly rare. Chandler<sup>1</sup> and Maplestone,<sup>2</sup> on the other hand, reported the incidence varying from 9 to 25 per cent in sheep- and goat-raising localities in India, while Jimbo<sup>3</sup> found 32.3 per cent of 1,215 stools examined at his clinic in Japan positive for *Trichostrongylus* infection. The species represented in these reports were presumably *Trichostrongylus colubriformis* in the former and definitely *Trichostrongylus orientalis* in the latter. Ransom's<sup>4</sup> predication in 1916, that the parasite may be found in man in continental United States, has not been fully realized. Among a few cases of infection with *Trichostrongylus colubriformis* reported, we may cite Sand-ground's<sup>5</sup> observation on his own experimental infection and the finding of a single male worm in a surgically removed appendix by Schenken and Moss<sup>6</sup> and identified by Faust.<sup>7</sup> In view of the paucity of human cases of *Trichostrongylus* infection, it appears to us that the present case is worth reporting. Furthermore, since there is a close resemblance between ova of this parasite and those of hookworms, a description of the characteristic ova seen in this case may help in the differential diagnosis of the two infections.

## REPORT OF A CASE

G. P., a laborer, aged 56 years, born in Greece, was admitted to Barnes Hospital with complaints of intermittent pain in the epigastrium and right upper quadrant, chronic constipation, dyspnea, productive cough, headache, ringing of left ear, and a loss of twenty-eight pounds of weight in a year. Physical examination: temperature was 37.5° C.; pulse, 80 per minute; respiration, 20; and blood pressure, 120/76. The patient was dyspneic and the posteroanterior diameter of the chest was increased. There were some inspiratory and expiratory wheezes heard chiefly at the apices of both lungs. The liver was tender, soft, and palpable three fingers below the costal margin. There was a suggestion of a mass extending down from the liver. Bladder dullness was percussed above the symphysis pubis. Laboratory examinations: red blood cell count, 5,120,000 per cubic millimeter; white blood cell count, 6,400; hemoglobin content, 13.2 Gm. per 100 c.c. Differential count was normal with no eosinophiles. Kahn test was positive 4 plus. Blood chemistry: nonprotein nitrogen, 56 mg. per cent; total serum proteins, 6.36 per cent; albumin, 4.26 per cent; globulin, 2.16 per cent. Icterus index was 6. Stool examination revealed the presence of a very few ova of *Trichostrongylus* sp., detectable only by the use of Willis' levitation technique,<sup>8</sup> and cysts of *Giardia lamblia*. The patient died shortly after admission. The clinical diagnosis of the attending physicians was generalized peritoneal carcinomatosis, substantiated by the examination of sediment of ascitic fluid, senile emphysema of lungs, and latent syphilis. Since autopsy was denied because of religious reason, the species of the parasite remains undetermined. An attempt to culture the organisms from the feces with powdered charcoal in order to secure typical larvae for definite identification was unsuccessful.

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## COMMENT ON THE CASE

Clinical observations by several workers, including Jimbo, seem to indicate that the severity of symptoms may be correlated with the number of the parasites localized in the small intestine. The scarcity of adult worms recovered at autopsy as well as the extremely small number of ova usually found in the feces of infected individuals led these investigators to conclude that the great majority of infected individuals are symptom free.

Since the symptoms exhibited by our patient were considered to be attributable to the diseases other than to the parasitic infection, no anthelmintics were administered by the attending physicians. Furthermore, no drugs are known to eradicate the parasite as exemplified in the reports of Jimbo, Sandground, and others. The history of the patient revealed that he had resided in the United States since he migrated from Greece in 1907. He had worked as a farm laborer at various times in the southern states. It is quite conceivable, therefore, that the infection might have been acquired by the accidental ingestion of the third-stage larvae from contaminated soil. According to Chandler, the infection probably occurs more frequently from the ingestion of vegetable matters contaminated with the larvae rather than by penetration through the skin. The possibility that the infection was originally acquired in Greece appears to be remote because of his thirty-six years of residence in this country. In the case of Sandground, the infection was reported to have persisted for as long as eight and one-half years.

## THE IDENTIFICATION OF OVA

The diagnosis of *Trichostrongylus* infection is based on the finding of the characteristic ova in the feces. As previously stated, the detection is usually difficult due to the small number of ova present in the feces, thus necessitating the use of a concentration technique. Moreover, because of a close resemblance of the ova to those of hookworms, the ova of *Trichostrongylus* were frequently mistaken for the ova of the latter parasite by casual observers. Since ova of various species of *Trichostrongylus* are indistinguishable one from the other, the definite identification of the species may be accomplished only by the recovery of adult worms following post-mortem examination or by the identity of the typical larvae in the feces-charcoal culture.

TABLE I  
MEASUREMENTS OF OVA *TRICHOSTRONGYLUS* SP.

LENGTH (MICRONS)	FREQUENCY	BREADTH (MICRONS)	FREQUENCY
81	1	40	3
82	1	41	5
83	2	42	4
84	3	44	6
85	3	45	1
86	6	47	1
87	3	49	1
88	2	51	1
90	1		
91	1		
94	1		
97	1		
86 (Average)		43 (Average)	

The ova of *Trichostrongylus sp.* found in the feces of the patient were transparent and elongated oval in shape. As compared with ova of hookworm, they are much longer and slightly wider. The measurements of twenty-five ova taken at random are tabulated in Table I.

As seen from Table I, the ova of *Trichostrongylus sp.* measure 81 to 97 microns in length by 40 to 51 microns in width, averaging 86 by 43 microns. The egg possesses a transparent shell membrane which is thicker and more greenish and lustrous than that of hookworms. While the ends of the ova are more pointed than those of hookworms, in a majority of them one end is somewhat pointed while the other is rounded, as shown in Fig. 1, resembling somewhat the shape of an elongated seedless grape. Maplestone likened it to a hen's egg, while Jimbo compared it to an elongated persimmon when viewed laterally. In some instances the pointed end appears slightly thicker than the



Fig. 1.—Egg of *Trichostrongylus sp.* recovered by the use of Willis levitation technique ( $\times 350$ ).

nonpointed end. Ova in freshly voided stool present an advanced stage of segmentation, usually sixteen to twenty-four morula stage of embryonation in staphylococcus-like arrangement. This difference, together with the characteristics described, may be used in differentiating the ova from those of hookworms.

In addition, ova of *Haemonchus contortus*, the parasite of sheep and goats and very rarely of man, possess morphologic characteristics which are indis-

tinguishable from those of *Trichostrongylus*. Differentiation, therefore, should be made by identifying adult worms or the third-stage larvae.

It is of interest to note in this connection that ova of *Strongyloides stercoralis*, which may rarely appear only in case of profuse diarrhea or following catharsis, may also be mistaken for ova of *Trichostrongylus*. As a rule, they may be differentiated from the latter by being smaller and by a thinner shell membrane which is less greenish in color and less lustrous. Moreover, in freshly voided stool *Strongyloides* ova contain embryos ready to be hatched. Ova of *Heterodera radicola*, which may temporarily appear in feces as result of ingestion of parasitized vegetable materials, require careful scrutiny. These ova may be distinguished from ova of *Trichostrongylus* by unilateral flattening or slight concavity with rounded ends, though distorted ova of the latter may lead one to an erroneous diagnosis. This difficulty may readily be overcome by repeatedly examining the feces following a vegetable-free diet.

In view of the possibilities of inaccurate diagnosis, the careful examination of the ova of these parasites is of utmost importance due to the fact that many individuals who do not respond to antihookworm therapy may be those passing only ova of *Trichostrongylus*. That mixed infections of hookworm and *Trichostrongylus* frequently occur has been verified by the report of Jimbo quoted by Koizumi.<sup>9</sup> Moreover, inaccurate diagnosis may lead one to an erroneous evaluation on the efficacy of drugs for the eradication of hookworm infection.

In areas where hookworm infections are prevalent, therefore, attention should be called to the necessity of carefully conducted examination. The larger size, unique shape, and advanced stage of segmentation at the time of deposition of ova of *Trichostrongylus* can hardly fail to attract the attention of those who keep them in mind. This will eventually result in uncovering cases of *Trichostrongylus* infections heretofore either unrecognized or incorrectly diagnosed.

#### SUMMARY

1. A case of *Trichostrongylus* infection is presented with the description of the characteristic ova found in the feces.

2. Because of the resemblance of ova of several parasites to those of *Trichostrongylus*, differential criteria are emphasized to facilitate the differentiation especially from hookworm ova, with which they may be confused by inexperienced observers.

3. The definitive identification of species of strongylate nematodes can be made only by the recovery of the adult worms or the third-stage larvae in culture, since the differentiation on the basis of morphology of ova alone is an impossibility.

4. An immediate need of more careful examination of stools is stressed particularly in localities where endemicity of hookworm infection exists.

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## PHOTOSENSITIVITY AS A CAUSE OF FALSELY POSITIVE CEPHALIN-CHOLESTEROL FLOCCULATION TESTS

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SINCE its introduction by Hanger<sup>1</sup> in 1939, the cephalin-cholesterol flocculation test has been widely used as a test of liver function, particularly as a method for detecting the presence of active parenchymatous disease of the liver. However, a disturbing difference in the sensitivity of the reaction has been reported by a number of observers<sup>2-6</sup> and found in our own experience.

This variability in the reaction was recently evidenced by two reports of attempts to design a dilution method for this test. Bruger<sup>4</sup> reported that in his experience the use of sera in various dilutions enabled him to estimate and follow the severity of the hepatic damage. Shortly thereafter Mirsky and Brecht,<sup>5</sup> using the method outlined by Bruger, reported that normal sera were consistently flocculated when used in dilute solution.

Neeffe and Reinhold<sup>6</sup> have recently demonstrated that photosensitivity may be a cause of falsely positive cephalin-cholesterol flocculation reactions. They have emphasized the importance of standardizing the technique if the results obtained by various laboratories are to be comparable. In attempting to set up standard conditions, we have confirmed the experience of Neeffe on the importance of light in causing falsely positive reactions.

Duplicate tests using the sera of ten normal individuals were made; one tube was kept in total darkness and the other placed outside a laboratory window in direct sunlight. Hanger's method with Difco cephalin-cholesterol mix was used. The results obtained are given in Table I.

The demonstration of the role of sunlight in initiating falsely positive cephalin-cholesterol flocculation tests necessitates that a standardized procedure be adopted if the results obtained by various laboratories are to be comparable. Duplicate samples of five normal sera were tested in an entirely dark cupboard, in direct sunlight, and in a small cupboard containing a 100-watt frosted electric bulb. From the data given in Table II it is evident that both sunlight and artificial light will give falsely positive reactions.

To determine whether the increased temperature induced in sera when they stood in sunlight altered the sensitivity of the test, duplicate specimens were warmed in a water bath at 40° C. for four hours and at 70° C. for thirty minutes (Table III). The results indicate that the falsely positive results are not due to heat. It is interesting to note that in the samples heated to 70° C. for one-half hour only slight flocculation could be obtained in one specimen and none in the other three after standing inside a window for three days. Perhaps alteration of some protein factor at the higher temperature may be responsible for this reaction.

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The patients used in this study were obtained through the courtesy of Dr. A. H. Colwell and the attending staff of the Presbyterian Hospital of the University of Pittsburgh.

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TABLE I

SERUM	DIRECT SUNLIGHT		DARKNESS	
	2 HR.	24 HR.	2 HR.	24 HR.
1	2+	3+	0	0
2	1+	3+	0	±
3	2+	4+	0	0
4	2+	3+	0	0
5	1+	3+	0	0
6	2+	4+	0	0
7	3+	4+	0	0
8	2+	3+	0	0
9	1+	4+	0	±
10	2+	4+	0	0

TABLE II

SERUM	DARKNESS 24 HR.	SUNLIGHT ½ HR.	SUNLIGHT 6 HR.	ARTIFICIAL LIGHT 24 HR.
1	0	0	3+	2+
2	0	0	2+	2+
3	0	±	4+	4+
4	0	0	2+	4+
5	0	0	2+	4+

TABLE III

SERUM	DARKNESS 24 HR.	SUNLIGHT 4 HR.	ARTIFICIAL LIGHT 24 HR.	4 HR. 40° C.	½ HR. 70° C.
1	0	3+	4+	0	0
2	0	2+	4+	0	0
3	0	2+	3+	0	0
4	0	4+	4+	0	0

TABLE IV

SERUM	CONDITION	DARK		LIGHT		
		24 HR.	2 HR.	6 HR.	12 HR.	24 HR.
1	Normal	0	0	1+	2+	3+
2	Normal	0	0	1+	2+	3+
3	Normal	0	0	0	2+	3+
4	Normal	0	0	0	1+	3+
5	Normal	0	0	1+	2+	4+
6	Normal	0	0	1+	2+	3+
7	Normal	0	0	0	2+	3+
8	Normal	0	0	0	1+	3+
9	Normal	0	0	1+	2+	3+
10	Normal	0	0	0	2+	3+
11	Thrombosis cent. vein	0	0	0	2+	3+
12	Disseminating lupus	0	0	0	2+	3+
13	Appendicitis	0	0	0	2+	3+
14	Cerebral arteriosclerosis	0	0	1+	2+	3+
15	Obstructive jaundice	0	0	2+	3+	4+
16	Obstructive jaundice	0	0	0	1+	3+
17	Obstructive jaundice	0	0	1+	2+	3+
18	Hepatitis	3+	2+	4+	4+	4+
19	Hepatitis	4+	2+	4+	4+	4+
20	Hepatitis	3+	1+	2+	3+	4+
21	Hyperthyroid crisis	0	1+	1+	2+	3+
22	Portal cirrhosis	0	2+	3+	4+	4+
23	Portal cirrhosis	0	1+	3+	4+	4+
24	Portal cirrhosis	0	1+	2+	3+	4+
25	Wilson's disease	0	1+	2+	3+	4+
26	Secondary carcinoma of liver	0	1+	2+	3+	4+
27	Multiple myeloma	0	1+	2+	3+	4+

In view of the demonstration of the photosensitivity of the cephalin-cholesterol flocculation test, it has become our practice to run duplicate specimens: one sample read after twenty-four hours in darkness and one read at two, six, twelve, and twenty-four hours in a small (24 by 18 by 16 inches) cupboard containing a 100-watt light bulb. In Table IV are summarized our results to date with this procedure. From these data the impression is gained that the serum from individuals with hepatic disease, although giving a negative cephalin flocculation reaction in the dark, may show abnormal sensitivity to light. Attention is called particularly to Cases 21 to 27 in which hepatic damage was present; the cephalin flocculation reaction in these cases was negative in the dark although showing abnormal sensitivity to light as compared to the controls (Cases 1 to 14).

Obviously if the results obtained by various laboratories are to be comparable, standardization of the light exposure is necessary. In our experience, placing the test tubes in a small cupboard containing a 100-watt light bulb, as noted in the preceding paragraph, has proved satisfactory.

#### CONCLUSIONS

The photosensitivity of the cephalin-cholesterol flocculation reaction has been confirmed. Since this reaction occurs in artificial light as well as in sunlight, it is not due to any property peculiar to the latter. It is not due to heat generated in the sample by light.

It is suggested that it may be worth while to study the photosensitivity of this reaction in patients with hepatic disease, and a simple method of doing this is given.

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# THE KAHN TRIPLE QUANTITATIVE VERIFICATION TECHNIQUE IN THE SEROLOGY OF MAL DEL PINTO (PINTA)

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MENK (1926) found that 74.5 per cent of the sera obtained from sixty-seven patients with mal del pinto, gave a positive reaction with the Wassermann test; 17 per cent were negative and 8.5 per cent were anticomplementary. Gonzalez Herrejon (1927) studied the blood of twenty-three patients with pinta and found twenty-one to be Wassermann positive and two Wassermann negative. Further observations have confirmed the high percentage of positive Wassermann, Kahn, Müller, Hinton, and Chediak tests in patients with mal del pinto. León y Blanco (1940) demonstrated that when the disease becomes generalized, the Wassermann, Kahn, and Müller tests are positive to the extent of 87.56 per cent with a maximum of 97 per cent in the late stages of the disease. Escobar (1940), Gonzalez Guzman (1940), and Briceño Rossi (1943) demonstrated that the Kahn verification test (differential temperature technique; Kahn, 1940) is positive in pinta as it is in syphilis.

In this paper we present the results of the Kahn triple quantitative technique (Kahn, 1942, 1943), which the author considers to have special specific value in the serology of syphilis. Also we present the results of other serologic experiments obtained with sera of patients with mal del pinto.

## MATERIAL AND METHODS

Different samples of blood from different localities of the States of Guerrero, Michoacán, and Nayarit were studied. Table I shows the type of the disease in each patient and those patients who were previously treated with arsenic compounds.

The triple quantitative technique for verification reaction was performed using three quantitative procedures: in the first one the serial dilutions were made with distilled water; in the second, the dilutions were made with 0.9 per cent saline solution; and in the third, with 2.5 per cent saline. The Kahn antigen used in all the experiments was the Difco antigen. The standard Kahn test was previously performed in every serum studied.

The globulin and the pseudoglobulin fractions of the blood sera of several patients with pinta were separated by the ammonium sulfate and dialysis method (Wadsworth, 1939). On each one of these fractions the Kahn standard test was performed.

## RESULTS

The results are shown in Table I. It can readily be seen that with the more concentrated saline solution used we obtained a stronger positive reaction. Each

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TABLE I  
RESULTS OF THE STUDIES OF SERA OF FORTY-SEVEN PATIENTS WITH MAL DEL PINTO

SERUM	TYPE OF PINTO	AGE (YR.)	TREAT-ED	KAHN STD.	TRIPLE QUANTITATIVE VERIFICATION TECHNIQUE				SERUM	TYPE OF PINTO	AGE (YR.)	TREAT-ED	KAHN STD.	TRIPLE QUANTITATIVE VERIFICATION TECHNIQUE			
					DIST. WATER	NaCl 0.9%	NaCl NIQUE	NaCl 2.5%						DIST. WATER	NaCl 0.9%	NaCl NIQUE	NaCl 2.5%
23	Pintids	12	Yes	++++	+++	++	++	+++	10	White	32	No	+	+++	+	+	-
5	Pintids	8	No	-	-	-	-	-	26	White	15	Yes	++	+	+	+	+
24	Pintids	14	Yes	++++	+	+	+	+	29	White	27	Yes	++	+	+	+	+
30	Pintids	8	No	-	-	-	-	-	32	White	20	Yes	+	+	+	+	+
1	Blue	16	No	-	-	-	-	-	42	White	14	Yes	++	+	+	+	+
25	Blue	16	Yes	++++	+	+	+	+	43	White	35	No	++	+	+	+	+
27	Blue	6	Yes	++++	+	+	+	+	48	White	40	Yes	++++	+	+	+	+
28	Blue	4	Yes	++++	+	+	+	+	50	White	40	Yes	++++	+	+	+	+
30	Blue	10	Yes	++++	+	+	+	+	52	White	7	Yes	++	+	+	+	+
31	Blue	17	Yes	++	+	+	+	+	55	White	22	No	++	+	+	+	+
40	Blue	19	Yes	++++	+	+	+	+	57	White	20	No	++	+	+	+	+
44	Blue	12	Yes	++++	+	+	+	+	73	White	30	No	++	+	+	+	+
45	Blue	45	No	++	+	+	+	+	74	White	40	No	++	+	+	+	+
51	Blue	35	Yes	++	+	+	+	+	75	White	36	No	++	+	+	+	+
53	Blue	9	Yes	++	+	+	+	+	2	White and blue	18	Yes	++	+	+	+	+
54	Blue	60	Yes	++	+	+	+	+	41	Red	30	Yes	++++	+	+	+	+
58	Blue	25	No	++++	+	+	+	+	46	Blue gray	20	Yes	++	+	+	+	+
78	Blue	34	No	++	+	+	+	+	49	Blue gray	20	Yes	++	+	+	+	+
3	White	30	No	++	+	+	+	+	47	Brown	22	Yes	++	+	+	+	+
4	White	30	No	++	+	+	+	+	21	Brown	4	Yes	++++	+	+	+	+
6	White	12	No	++++	+	+	+	+	79	Brown	7	Yes	++	+	+	+	+
7	White	10	Yes	++++	+	+	+	+	80	Brown	7	Yes	++	+	+	+	+
8	White	12	No	++	-	-	-	-	81	Brown	8	Yes	++	+	+	+	+
9	White	13	No	++	+	+	+	+				Yes	++	+	+	+	+

- = Negative.

- = Negative.

column in Table I represents the average flocculation intensity read in four Kahn tubes containing serum dilutions of  $\frac{1}{5}$ ,  $\frac{1}{10}$ ,  $\frac{1}{20}$ , and  $\frac{1}{40}$ , respectively.

The standard Kahn tests with the euglobulin and pseudoglobulin fractions separated from sera of patients with pinta were positive for both fractions.

#### SUMMARY

The different concentrations of sodium chloride from 0 to 2.5 per cent, applied in Kahn's triple quantitative verification technique, increase the intensity of the reaction in the sera of patients with mal del pinto.

The globulin and pseudoglobulin fractions of sera of these patients reacted in a similar way, both giving positive standard Kahn tests.

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## CLINICAL CHEMISTRY

### NITROGEN BALANCE ON A RESTRICTED CALORIC INTAKE

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**D**URING early studies on intravenous protein feeding with amino acids<sup>1</sup> we always tried to provide all of the energy requirements by giving sufficient glucose. To supply 1,600 calories parenterally means an intravenous injection lasting seven hours inasmuch as a normal adult can utilize only 60 Gm. per hour. Moreover, this would mean 4,000 c.c. of 10 per cent glucose or 8,000 c.c. of 5 per cent glucose. Because of the obvious practical difficulties, we began to question the necessity of supplying all of the energy requirements, during short periods of time at least. Quite to our surprise we found that positive nitrogen balance could be maintained in many of those patients even though the amount of glucose administered fell far short of that required for full energy needs. Obviously the rest of the calories were being obtained from tissue fat. In order to study this matter further, a series of experiments was carried out in dogs to determine how well nitrogen balance can be maintained on a restricted caloric but adequate protein intake.

#### PREVIOUS OBSERVATIONS

Lusk<sup>2</sup> states that under conditions of complete starvation 87 per cent of the caloric requirements are met by tissue fat, the rest coming from tissue protein, which seems to offer ample theoretical justification for relying on tissue fat for much of the caloric needs. Indeed, Rubner<sup>3</sup> was able to cut the caloric requirements by 33 per cent without disturbing the degree of nitrogen balance. On the other hand, Thomas<sup>4</sup> found that nitrogen balance could not be maintained on a pure protein diet; yet it is obvious that the Eskimos maintain health and growth on a diet which is largely protein with some fat but little or no carbohydrate. Block<sup>5</sup> studied nitrogen balance in three obese and three normal women for consecutive seven-day periods during which the caloric intake was reduced from 80 to 20 per cent of the basal requirement, although the protein intake was maintained at 1 Gm. or more per kilogram per day. Positive nitrogen balance was observed in twenty of twenty-four periods. Moreover, the weight loss in the obese patients was exactly as predicted on the basis that the adipose tissue furnished the calories not ingested in the diet. In a previous report from this laboratory<sup>6</sup> dogs were shown to retain much more nitrogen on isocaloric but inadequate diets in which 80 per cent of the calories were protein as compared with those in which 80 per cent of the calories were carbohydrate.

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## PRESENT OBSERVATIONS

Four healthy female mongrel dogs were selected for these experiments. They were prepared for easy catheterization by dividing the posterior vaginal wall. After healing had occurred they were observed two at a time. Previous to the beginning of each series of observations, the dogs had been on a full well-balanced diet and were well nourished.

The dietary intake during the experiment was rigidly controlled by gavage feedings. No other food was permitted except that water was allowed *ad libitum*. They were kept in metabolism cages for the collection of urine and were catheterized at the beginning of each period which in general lasted four days. The diet consisted of dextrimaltose and Amigen, which is an enzymic hydrolysate of casein and pork pancreas. Two different mixtures were prepared consisting of (a) 80 per cent carbohydrate and 20 per cent protein (as Amigen) and (b) 80 per cent protein (as Amigen) and 20 per cent carbohydrate. The two dietary constituents were mixed in a small quantity of water and given by gavage once a day. This procedure worked very well and provoked vomiting only twice in one animal and is the only occasion in which consecutive observations were not carried out (Table II). Otherwise, the experiments were without event, including the absence of diarrhea. The stools were collected in the early experiments but were found to be quite scanty and to comprise less than 5 per cent of the total nitrogen in the urine and were therefore discontinued.

The daily amount of food administered was set at either 50 calories per kilogram or 25 calories per kilogram, based on 4 calories per gram of carbohydrate as dextrimaltose and 3 calories per gram of Amigen. In calculating the caloric value of Amigen, its nitrogen content was used as the base; this was multiplied by 6.25, giving its presumed protein equivalent. Because Amigen contains only 12 per cent instead of the 16 per cent nitrogen in whole protein, a figure of 3 calories per gram of Amigen instead of the 4 calories for whole protein was obtained. Inasmuch as a figure of 3.7 calories per gram of Amigen was obtained by direct bomb calorimetry,<sup>7</sup> this figure may be incorrect. If the figure of 3.7 were taken instead of 3.0, the difference would involve the number of calories given in each group of experiments. Thus the caloric intake instead of being 50 calories in each animal would be 52 in the low protein and 58 in the high protein mixture. At a 25 caloric intake, the difference would be 26 as against 29. This slight discrepancy, however, would probably not influence the main findings even if this calculation were justified. Nitrogen was determined by a micro-Kjeldahl technique described by Sobel, Yuska, and Cohen.<sup>8</sup>

## EXPERIMENTAL FINDINGS

The observations made are recorded in Tables I to III. It will be noted that when the dietary intake consisted of 80 per cent carbohydrate and 20 per cent protein (as Amigen), nitrogen balance was achieved at a level of 50 calories per kilogram in only one of the two dogs. Moreover, when the caloric intake was cut in half in these dogs, a negative balance was found in each of the two animals (Table I). By contrast, positive nitrogen balance was obtained even at a 25 calories per kilogram intake in similar experiments in two other dogs in which the proportion was reversed so as to contain 80 per cent protein (as

TABLE I

NITROGEN (N) BALANCE ON AN INTAKE OF 80 PER CENT CARBOHYDRATE AND 20 PER CENT PROTEIN (AS AMIGEN)  
CALORIC INTAKE DURING PERIODS I TO III, 50/Kg.; DURING PERIODS IV TO VII, 25/Kg.

PERIOD (4 DAYS)	DOG I. (WEIGHT, 12.4 KG.)				DOG S (WEIGHT, 7.2 KG.)			
	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE
	CH (GM.)	N <sub>1</sub> (GM.)	N <sub>2</sub> (GM.)	N <sub>1</sub> -N <sub>2</sub> (GM.)	CH (GM.)	N <sub>1</sub> (GM.)	N <sub>2</sub> (GM.)	N <sub>1</sub> -N <sub>2</sub> (GM.)
I	500	20	24.88	-4.88	288	11.4	11.32	+0.08
II	500	20	20.92	-0.92	288	11.4	10.72	+0.68
III	500	20	20.16	-0.16	288	11.4	9.44	+1.96
Average per day				-0.49				+0.23
IV	250	10	14.04	-4.04	144	5.7	8.00	-2.30
V	250	10	11.64	-1.64	144	5.7	6.88	-1.18
VI	250	10	11.76	-1.76	144	5.7	6.96	-1.26
VII	250	10	11.72	-1.72	144	5.7	7.40	-1.70
Average per day				-0.57				-0.40

TABLE II

NITROGEN (N) BALANCE ON AN INTAKE OF 20 PER CENT CARBOHYDRATE AND 80 PER CENT PROTEIN (AS AMIGEN)  
CALORIC INTAKE DURING PERIODS I TO III, 50/Kg.; DURING PERIODS IV TO VI, 25/Kg.

PERIOD (4 DAYS)	DOG B (WEIGHT, 9.3 KG.)				DOG T (WEIGHT, 10.5 KG.)			
	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE
	CH (GM.)	N <sub>1</sub> (GM.)	N <sub>2</sub> (GM.)	N <sub>1</sub> -N <sub>2</sub> (GM.)	CH (GM.)	N <sub>1</sub> (GM.)	N <sub>2</sub> (GM.)	N <sub>1</sub> -N <sub>2</sub> (GM.)
I	93	59.52	51.12	+8.40	*105	67.2	72.04	-4.84
II	93	59.52	54.28	+5.24	*105	67.2	59.24	+7.96
III	93	59.52	56.72	+2.80	---	---	---	---
Average per day				+1.37				+0.39
IV	47	29.76	30.68	-0.92	53	33.6	34.52	-0.92
V	47	29.76	28.60	+1.16	53	33.6	32.40	+1.2
VI	47	29.76	27.40	+2.36	53	33.6	31.76	+1.84
Average per day				+0.22				+0.19

\*Periods not consecutive due to vomiting.

TABLE III

NITROGEN (N) BALANCE ON AN INTAKE OF 25 CALORIES PER KILOGRAM  
PERIODS I TO IV ON DOG T AND PERIOD V ON DOG B, INTAKE WAS 80 PER CENT CARBOHYDRATE  
AND 20 PER CENT PROTEIN (AS AMIGEN); PERIODS I TO IV ON DOG B AND PERIOD V ON  
DOG T, INTAKE WAS 20 PER CENT CARBOHYDRATE AND 80 PER CENT  
PROTEIN (AS AMIGEN)

PERIOD (4 DAYS)	DOG T (WEIGHT, 9.7 KG.)				DOG B (WEIGHT, 9.4 KG.)			
	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE
	CH (GM.)	N <sub>1</sub> (GM.)	N <sub>2</sub> (GM.)	N <sub>1</sub> -N <sub>2</sub> (GM.)	CH (GM.)	N <sub>1</sub> (GM.)	N <sub>2</sub> (GM.)	N <sub>1</sub> -N <sub>2</sub> (GM.)
I	194	7.6	14.60	-7.0	47	30.0	29.32	+0.68
II	194	7.6	11.16	-3.56	47	30.0	24.68	+5.32
III	194	7.6	9.12	-1.52	47	30.0	24.60	+5.40
IV	194	7.6	9.12	-1.52	47	30.0	26.48	+3.52
Average per day				-0.85				+0.93
V (5 days)	60	38.0	29.4	+8.6	235	9.4	15.75	-6.35
Average per day				+1.7				-1.27

Amigen) and 20 per cent carbohydrate (Table II). This same effect was observed more strikingly in the next two experiments in which the animals were placed immediately on a 25 calorie per kilogram intake. In one the proportion was 80 per cent carbohydrate and 20 per cent protein, which produced a persistent average negative balance of 0.85 Gm. per day for four consecutive four-day periods, whereas in an immediately following five-day period, in which the proportion was reversed, pronounced positive balance of 1.7 Gm. per day was achieved. In the other experiment in which the 25 calories consisted of 80 per cent protein (as Amigen) and 20 per cent carbohydrate, positive nitrogen balance of nearly a gram a day was consistently maintained in four consecutive four-day periods, whereas in an immediately following five-day period in which the proportion was reversed, a negative balance of 1.27 Gm. per day was observed (Table III).

#### COMMENT

From the findings herein presented it would seem that good nitrogen balance can be achieved by simply increasing greatly the proportion of nitrogen in the diet, even though the calorie intake is far from adequate. This has certain practical implications. For example, the necessity of supplying all of the energy requirements during short periods of intravenous feeding assumes less significance, and the surgeon is safely relieved of the practical difficulty of supplying as much glucose as was hitherto deemed necessary. It is probable that a relatively small amount of glucose will permit adequate utilization of protein for short periods of time at least. Undoubtedly the rest of the calorie needs are met by use of tissue fat.

These findings have a further implication in wartime in that members of the Armed Forces are not infrequently separated from their sources of supply and must for certain periods maintain themselves on a restricted ration, which leads to loss of body tissue. Because part of the calorie needs may be met by tissue fat which contributes little to physiologic performance, it is much more important to maintain protein tissue which is of great physiologic significance. From this it may be inferred that priority should be given to protein rather than to calorie intake. Because the present observations show that positive nitrogen balance can be maintained on a low calorie intake when sufficient protein is provided, it would seem obvious that such a ration would result in greater physiologic efficiency than one consisting largely of carbohydrate and is associated with a negative nitrogen balance. In other words, with a restricted diet consisting purely of carbohydrate and protein, a better metabolic and physiologic result will follow when the proportion allotted to carbohydrate is reduced and that allotted to protein is increased, in contrast to the normal distribution in a well-balanced diet.

#### SUMMARY

In dogs on restricted isocaloric intake, positive nitrogen balance can be maintained when the proportion of protein to carbohydrate is 4 to 1 but not 1 to 4, respectively. The practical implications of these findings in regard to the desirability in restricted diets of giving priority to protein over calorie needs are discussed.

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# LIMITATIONS OF THE CORRELATION BETWEEN THE RED CELL VOLUME AND HEMOGLOBIN OF BLOOD DURING PREGNANCY

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IT HAS been rather generally accepted that the red cell volume of the blood and the hemoglobin are proportional and that one value may be checked by the other. A number of exceptions discovered among pregnant patients led us to undertake an investigation of the true value of this possible correlation during the course of pregnancy.

In a series of consecutive determinations made on seventy-five pregnant women upon admission to the prenatal dispensary, using oxalated blood, we found Pearson's correlation to be 0.677 between the red cell volume and the hemoglobin. In a second series of thirty-seven patients, using heparinized blood, we found a correlation of 0.747. This means that while there is a definite correlation, of proved significance, in either series, one is not justified in predicting one value from a determination of the other, even when using heparinized blood. This is illustrated in Fig. 1, which shows the individual distribution of the hemoglobin-cell volume relations. The two lines indicate true proportionalities, based on the hemoglobin and cell volume averages, and coincide with those drawn to indicate the tendency toward proportion, in either series, as shown by the distribution of the individual findings. It is clear, from Fig. 1, that while a certain correlation does exist, individual determinations show too great a variance, in both directions, for it to be applicable in individual cases.

The cell volumes were found by spinning the oxalated or heparinized blood at high speed until there was no further packing of the cells; then the volume of the latter is given in per cent of the volume of the whole blood. For the oxalated series we found a mean value of 33.4 per cent, with a standard error of 0.41. The computed range, from these figures, to cover individual determinations, is from 22.7 to 44.1 per cent. With the heparinized blood the mean value was 38.5 per cent, with a standard error of 0.64, giving a computed range of 26.9 to 50.2 per cent.

Hemoglobin was determined by the Fisher electrophotometer, using the method described by Evelyn,\* 50 cu. mm. of blood in 10 c.c. of water, with 1 drop of strong ammonium hydroxide added immediately before reading, using a 525 millimicron filter. For the combined series of 112 determinations, we found a mean of 11.43 Gm. hemoglobin per 100 c.c. of blood, with a standard error of 0.11. The computed range for individual determinations would be from 7.8 Gm. to 15.0 Gm. per 100 c.c.

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\*Evelyn, K. A.; Determination of Oxyhemoglobin, J. Biol. Chem. 115: 63, 1936.

## SUMMARY

Correlations of 0.677 and 0.747 were found between the red cell volume and hemoglobin of blood from pregnant women, using potassium oxalate and heparin, respectively, as anticoagulants. Variations were found in about 25 per cent of the cases, in both directions, as shown in Fig. 1.

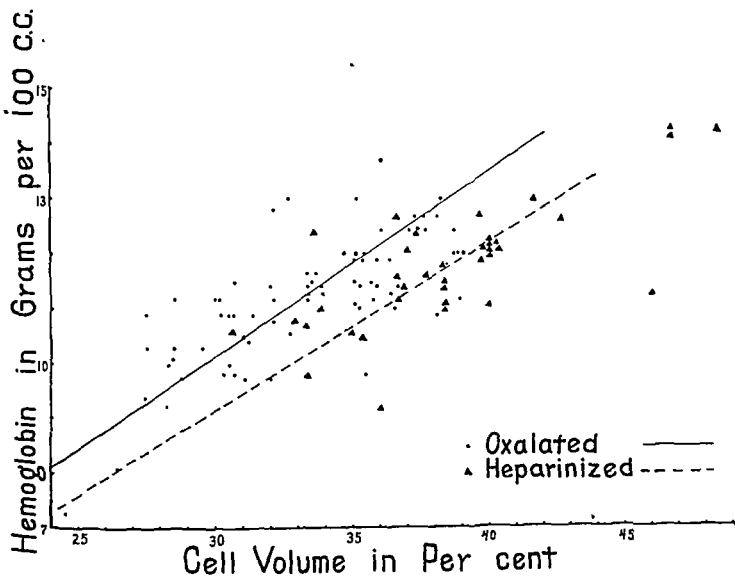


Fig. 1.—Distribution of the individual hemoglobin-cell volume determinations on pregnant women.

Mean values, with computed ranges to cover individual findings, were established for the red cell volumes and hemoglobin concentration in blood from pregnant women.

# LABORATORY METHODS

## A METHOD FOR THE COLORIMETRIC DETERMINATION OF POTASSIUM IN BIOLOGIC PRODUCTS

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IN UNDERTAKING a study on the effect of protein and amino acid deficiency states on the mineral metabolism of the human being and experimental animals, it became necessary to find a satisfactory, yet rapid, procedure for the estimation of potassium in the urine and tissue digests. Our experiments with a number of the many procedures available for this analysis demonstrated that on modification the colorimetric technique reported by Blanchetière and Pirlot<sup>1</sup> was extremely well suited to our purposes. This method is based on the measurement of potassium as the green color produced by the basic carbonate of cobalt which is obtained by transforming the insoluble potassium sodium cobaltinitrite into the respective chlorides of the complex cations and treatment of the mixture with bicarbonate. Our modification of the procedure involved an improvement in the over-all accuracy by establishment of optimal precipitation conditions for the cobaltinitrite complex and increased speed of operation achieved by the elimination of what proved to be a long and unnecessary dehydration period for the conversion of the cobalt complex into the chlorides by the use of concentrated hydrochloric acid. The adequacy of these modifications was demonstrated by the quantitative recovery of potassium added to urine specimens and tissue digests.

In order to obtain base-line data for our subsequent studies on human subjects fourteen twenty-four hour urine specimens from seven normal adult males maintained on unrestricted normal diets were analyzed for potassium and nitrogen content. These were found to contain from 2.92 to 4.76 Gm. of potassium with the nitrogen potassium ratios varying from 2.51 to 4.05 for the series.

Application of the method to human serum and red blood cells yield values which compare favorably with those reported in the literature. Analysis of neutralized acid digests of eight whole immature rats (70 to 125 Gm.) showed the potassium content to be  $0.61 \pm 0.04$  per cent.

### EXPERIMENTAL

#### *Reagents.—*

*Sodium cobaltinitrite reagent:*<sup>2</sup> To a solution of 25 Gm. of cobalt nitrate dissolved in 62.5 c.c. of 20 per cent acetic acid (by volume) in a 1 liter Erlenmeyer flask are cautiously added 210 c.c. of a solution of sodium nitrite prepared by dissolving 120 Gm. of the salt in 180 c.c. of water. After the vigorous evolution of nitrogen oxide gases which ensues has subsided, the remainder

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of the gases are removed by drawing air through the solution. The reagent is then filtered and stored in the icebox where it keeps well for three or four months.

*Ethyl alcohol*: 30 per cent solution by volume.

*Hydrochloric acid*: 90 per cent solution by volume.

*Potassium bicarbonate*: Saturated solution.

*Hydrogen peroxide*: 3 per cent solution prepared by 1:10 dilution of Superoxol.

*Potassium standard*: 94.8 mg. of oven-dried (100°) potassium chloride are dissolved in 100 c.c. of distilled water; 1 c.c. of this solution is equivalent to 0.5 mg. of potassium.

#### PROCEDURE

*Preservation of Urine*.—Twenty-four hour specimens were collected in brown bottles containing 50 c.c. of 15 per cent HCl (by volume) and 1 c.c. of 10 per cent alcoholic thymol and were made to a uniform volume of 2 liters before removal of the sample for potassium determination. It has been found that under these conditions the potassium content of the specimens remains unchanged after storage for one week or more at room temperature.

*Method*.—To 1 or 2 c.c. of urine, or samples (pH 5-6) containing not more than 3 mg. of potassium, in a 15 c.c. graduated conical centrifuge tube are added 2 c.c. of the cobaltinitrite reagent and distilled water to make a final reacting volume of 5 c.c. After the mixtures have been allowed to stand for at least two hours at room temperatures, the tubes are centrifuged for ten minutes at 3,000 r.p.m. and the supernatant solutions discarded by careful decantation. The yellow precipitates are washed twice by successive resuspension in 5 c.c. of 30 per cent ethanol, centrifugation, and decantation of the supernatant fluid. The tubes are inverted on to filter paper and drained for ten or fifteen minutes; then 0.5 c.c. of 90 per cent hydrochloric acid is added to each sample and the precipitates are dissolved and nitrogen oxide gases driven off by heating gently over a Fischer microburner. The blue test solutions are cooled in tap water, and on the addition of 0.5 c.c. of 3 per cent hydrogen peroxide a deep pink color appears. Now saturated potassium bicarbonate solution is added dropwise until effervescence ceases and the solutions turn to a deep emerald green color. The samples are all made to the 10 c.c. mark with bicarbonate solution and read in the Klett-Summerson photoelectric colorimeter using the S-60 filter. As a check on the satisfactory performance of these operations, 1 c.c. and 5 c.c. aliquots of the potassium standard are run simultaneously with each set of urine samples. Since the color intensity shows no tendency to fade in two or three hours, it is possible to perform as many as twelve duplicate determinations in each run without danger of error from this source.

*Calculations*.—The amount of potassium in the samples can be readily calculated from the following formula:

$$\frac{\text{Reading of sample}}{\text{Reading of standard}} \times \text{mg. of K in standard} = \text{mg. of K in sample}$$

Or, since the color reaction obeys Beer's law, the potassium content of a given sample can be estimated directly from a previously prepared calibration curve.



## RESULTS

In order to assess the quantitative effect of the modifications made in the reaction conditions of the method, suitable aliquots of the potassium standard were submitted to the manipulations previously described. The linear relationship of the color intensity to the amount of potassium which is obtained by the use of the method (Fig. 1) is interpreted as evidence of the suitability of the modifications and validity of Beer's law for the color reaction.

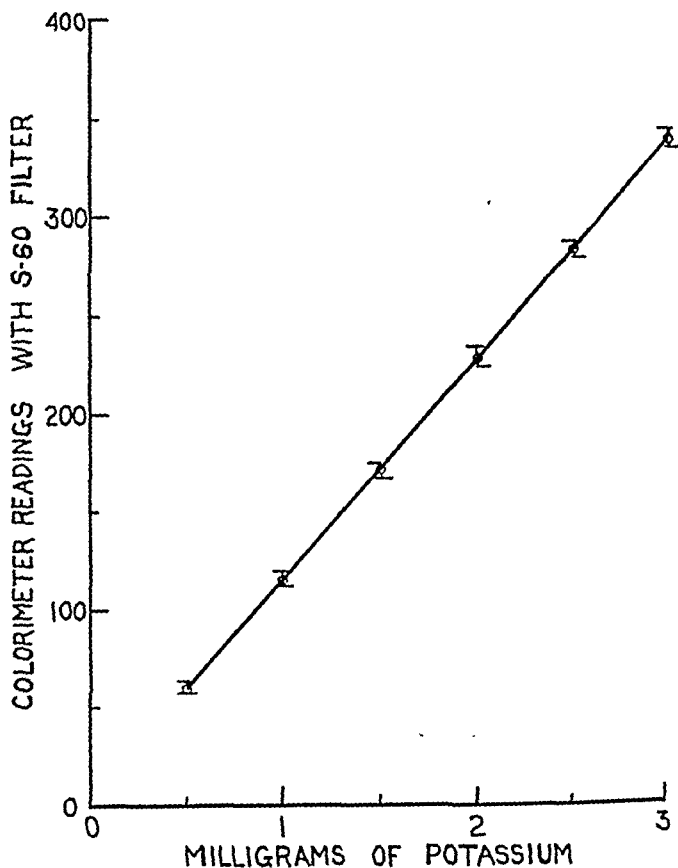


Fig. 1.—Relation of color intensity to amount of potassium. Each point represents the average of ten determinations. The bars above and below each point indicate the deviation range of the readings.

The applicability of the procedure to the urine was indicated by the quantitative recovery of added potassium (Table I).

The results of the nitrogen<sup>3</sup> and potassium analysis of twenty-four hour specimens of seven normal adults on unrestricted normal diets are shown in Table II. When due consideration is given to the qualitative and quantitative variations of the food intake which prevailed, the N/K ratio would not seem to fluctuate too greatly either for the individual or the group.

The analyses of 1 and 2 c.c. samples of blood serum from six different normal children subsequent to ashing in a muffle furnace for twenty-four hours at 500° C. gave values ranging from 14.4 to 21.8 mg. per 100 c.c. The potassium

content of adult human erythrocytes was determined by ashing 200 to 500 mg. samples of carefully washed and dried cells in the muffle furnace and found to be 0.44 per cent. These figures are well within the range of those reported by other investigators.<sup>4</sup>

TABLE I  
RECOVERY OF ADDED POTASSIUM

SAMPLE	POTASSIUM ADDED (MG.)	POTASSIUM FOUND (MG.)	RECOVERY OF ADDED POTASSIUM (PER CENT)
Urine A, 1 c.c.	0	1.33	
Urine A, 1 c.c.	0.53	1.90	102.0
Urine B, 1 c.c.	0	1.78	
Urine B, 1 c.c.	0.53	2.30	99.5

TABLE II  
DAILY OUTPUT OF POTASSIUM AND NITROGEN OF NORMAL MALES ON NORMAL DIET

SUBJECT	BODY WEIGHT (KG.)	FIRST WEEK			SECOND WEEK		
		TOTAL K (GM.)	TOTAL N (GM.)	N/K RATIO	TOTAL K (GM.)	TOTAL N (GM.)	N/K RATIO
4	79.5	3.30	12.95	3.92	4.26	11.48	2.69
5	63.6	2.92	10.75	3.68	3.22	10.60	3.29
6	65.9	3.16	11.40	3.61	3.23	10.90	3.38
7	77.4	3.90	10.48	2.68	4.24	10.60	2.51
8	71.9	3.57	12.40	3.48	3.56	12.90	3.62
9	100.0	4.76	17.30	3.63	3.82	14.91	3.91
10	79.5	4.76	17.20	3.62	3.34	13.54	4.05

# COMMENT

The accuracy of potassium determinations based on gravimetric or titrimetric or colorimetric elaborations of the cobaltinitrite precipitation has been the subject of much discussion. The errors inherent to the gravimetric methods arise from variations in potassium content and solubility of the complex. Hubbard<sup>5</sup> and Jendrassik<sup>6</sup> have described schemes to overcome the variability in composition. Solubility errors prompted Breh and Gaebler<sup>6</sup> to precipitate potassium as silver potassium cobaltinitrite which is presumably less soluble than the sodium analog. In spite of these modifications, unanimity of opinion on the validity of gravimetric data does not seem to have been reached as yet. Inasmuch as in our method the color readings are made with reference to a potassium standard which has been submitted to operations identical to those of the unknown, it is not necessary to consider the effect of composition variations of the complex or to correct for its solubility. That these factors remain constant in our procedure can be adduced from the reproducibility of the calibration curve and recovery test data.

The titrimetric estimation of potassium by the use of acid permanganate has been criticized principally on the score that the precipitates may contain organic substances which would result in high values. Devices to circumvent this difficulty have been described by Hubbard<sup>5</sup> and Leulier,<sup>8</sup> but they are laborious and of doubtful efficacy.

Of the many inorganic and organic color reactions reported in the literature for the assay of potassium in terms of the cobalt or nitrite content of the complex, that of Blanchetière and Pirlot<sup>1</sup> was selected by us because it is the least

affected by variations in reagents quantities and yields the most stable color. The use of artificial standards is obviously not to be recommended as these would introduce the composition and solubility errors discussed below.

Calculations from Clark's<sup>9</sup> data show that the adult male retains from 200 to 180 mg. of potassium per day of an average intake of 2.47 Gm. of potassium. The experiments of Loeb and associates<sup>10</sup> confirm these figures and further reveal that the retention is not greatly affected by an increase in the potassium input. In view of the relatively small potassium requirements of the human adult, it is obvious from our excretion data that the average American diet supplies an abundance of potassium. It is of further interest to note that our potassium excretion values are in good agreement with the calculated average potassium content of the American daily diets.<sup>4</sup>

#### SUMMARY

A simple and accurate colorimetric method for the estimation of potassium in biologic materials has been described. Application of the method to urine of seven normal adult males on normal diets showed that the daily potassium output varies from 2.92 to 4.76 Gm. The analyses of human blood serum and red blood cells gave values which are in accord with those found in the literature.

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# THE OCCURRENCE OF HIGH BLOOD BLANKS IN THE COLORIMETRIC DETERMINATION OF SULFONAMIDES

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HOLBOURN and Pattle<sup>1</sup> have recently claimed that in their modification of the dimethyl- $\alpha$ -naphthylamine method<sup>2</sup> for estimating sulfanilamide it is not necessary to remove the excess nitrite. Under our conditions it has been found, however, that this method may give very high blank values for normal blood (rat, rabbit, and beef), especially when hydrolyzed with HCl. When high blood dilutions, such as 1:200, are used, the blank values for normal filtrates hydrolyzed with HCl have reached 50 mg. per cent, expressed as sulfanilamide.

A purple color is produced, the intensity of which increases indefinitely. This is apparently due to impurities in the coupling agent, for Marshall<sup>3</sup> states: "A high blank value may be obtained with certain samples of dimethyl- $\alpha$ -naphthylamine due to contamination with  $\alpha$ -naphthylamine." We have found that destruction of the excess nitrite with urea, or with ammonium sulfamate as in Marshall and Litchfield's<sup>2</sup> method, results in relatively steady readings and low blanks.

*Method.*—Normal blood was hemolyzed in distilled water and precipitated with trichloroacetic acid at a final concentration of 3 per cent. Hydrolysis was carried out by heating the filtrates for one hour in the water bath in the presence of 1 c.c. of 4 *N* HCl or  $H_2SO_4$  per 20 c.c. of filtrate. Readings were taken in a photoelectric colorimeter with a green filter ten and fifteen minutes after the addition of the coupling agent. The various methods were standardized against sulfanilamide, using the ten-minute readings.

The coupling agents, dimethyl- $\alpha$ -naphthylamine and N-(1-naphthyl)-ethylenediamine dihydrochloride, were obtained from the Eastman Kodak Co. All other chemicals were of reagent grade. In the modified Holbourn and Pattle method, urea was added as 0.5 c.c. of 40 per cent solution, and ammonium sulfamate as 0.5 c.c. of 0.5 per cent solution, three minutes after the addition of nitrite. After a further two-minute period the coupling agent was added.

*Results.*—Typical of our results with various blood samples are the values given in Table I for blanks on normal rabbit blood diluted 1:25. The values are expressed as equivalent sulfanilamide concentrations in the filtrates, reagent blanks having been deducted from the blood blanks.

When dimethyl- $\alpha$ -naphthylamine is used and the excess nitrite is destroyed, the blank values are low and the readings are virtually constant. The values compare favorably with those obtained in the Bratton and Marshall method<sup>4</sup> using N-(1-naphthyl)-ethylenediamine. Lee, Hannay, and Hand<sup>5</sup> have claimed that in this latter method the sulfamate may be omitted if alcohol is added. They recommend the use of  $H_2SO_4$  for hydrolysis, and Table I shows that by this method the blank values are extremely low; when HCl is used, however, the

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blanks are high. We have observed that steady readings are not obtained unless at least 0.2 mg. per cent of sulfanilamide is present. However, the errors are small when ten minutes is taken as the standard time, and the absence of gas bubbles makes this method very convenient.

TABLE I  
BLANK VALUES ON NORMAL RABBIT BLOOD DILUTED 1:25  
(EXPRESSED AS  $\mu\text{G.}$  SULFANILAMIDE PER 100 C.C. FILTRATE)

METHOD	REAGENT		BLOOD FILTRATE		HYDROLYZED FILTRATE	
	10 MIN.	15 MIN.	10 MIN.	15 MIN.	10 MIN.	15 MIN.
Holbourn and Pattle <sup>1</sup>	54	65	47	64	73	93
Holbourn and Pattle <sup>1</sup> plus urea	41	38	2	3	5	5
Holbourn and Pattle <sup>1</sup> plus sulfamate	38	36	2	1	4	5
Bratton and Marshall <sup>4</sup>	5	5	3	3	4	4
Lee, Hannay, and Hand <sup>5</sup>	26	24	-2	-3	0	-2
Lee, Hannay, and Hand <sup>5</sup> hydrolyzed with HCl	--	--	--	--	24	23

#### SUMMARY

In the colorimetric determination of sulfonamides with dimethyl- $\alpha$ -naphthylamine and with N-(1-naphthyl)-ethylenediamine, normal blood filtrates may give very high blank values unless the excess nitrite is destroyed with urea or sulfamate. The blanks are especially high when blood filtrates are hydrolyzed with HCl.

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## NEW TYPE OF NEEDLE HOLDER\*

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IN DOING venepunctures at the Red Cross Blood Donor Center a small quantity of novocain is injected with a sterile hypodermic needle before the venepuncture needle is inserted. Where 900 to 1,000 donors are bled daily a large number of hypodermic needles are required since a fresh sterile needle is used for each donor. To have these needles readily available for use has constituted quite a problem. Many of the Blood Donor Centers use individual test tubes

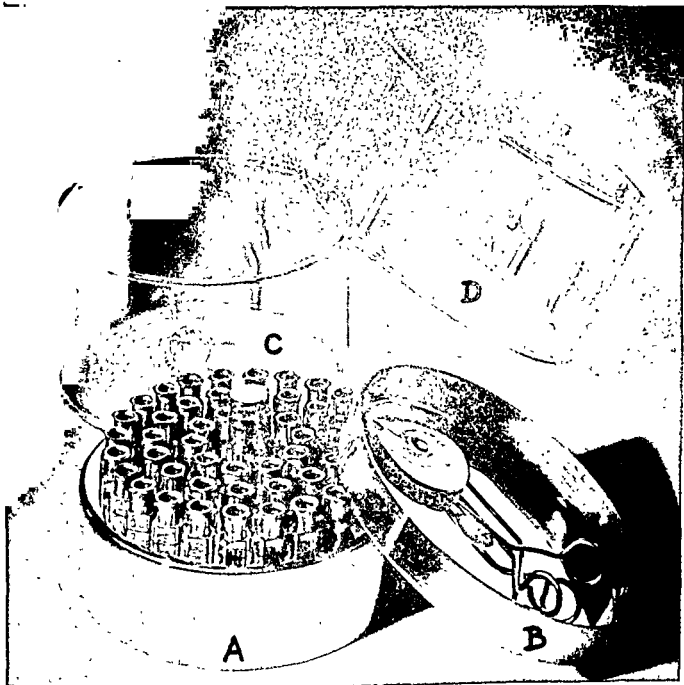


Fig. 1.—A, Needle holder 25 by 35 mm.; B, spring lock cover; C, crystal dish 90 by 50 mm.; D, crystal dish cover 100 by 50 mm.

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\*This needle holder was made for the Chicago Blood Donor Center by The Helmeo Corporation, 1215 West Fullerton Ave., Chicago 14, Ill.

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for each needle, but that involves a great deal of work both in preparing the needles for autoclaving and in removing the needles from the test tubes.

At the Chicago Blood Donor Center cork needle holders were used, each needle holder containing 40 needles and being covered by a crystal dish 100 by 50 mm. This was a time-saver but had the disadvantage that after repeated autoclaving the cork would deteriorate and cause plugging of many of the needles. Also, the cost of discarded cork holders and the increasing difficulty in securing the cork were additional factors in prompting us to search for a method to overcome these objections. As a result a new type of needle holder has been developed.

The new needle holder (Fig. 1), which is now in use at the Blood Donor Center of the Chicago Chapter of the American Red Cross, was constructed of spun copper, chromium plated, with a special brass supporting knob with a snap-on lock feature accommodating the spring lock cover. This type of needle holder offers many advantages in that it is light, durable, and easily sterilized. It holds 50 needles size 25-G,  $\frac{5}{8}$  of an inch in length.

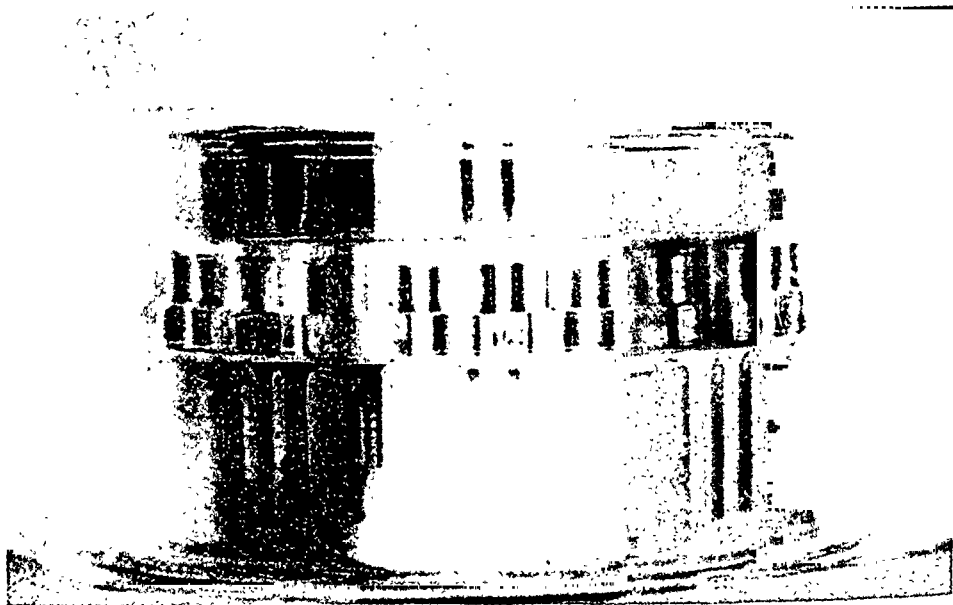


Fig. 2.—Needle holder assembled.

The chromium needle holder (Fig. 1, A) is placed in a crystal dish (Fig. 1, C) 90 by 50 mm. The needles are placed in individual perforations with points down and covered with a chromium-plated lid with a spring lock (Fig. 1, B) which holds the needles in position, and the entire holder is covered with a crystal dish 100 by 50 mm. (Fig. 1, D) and then autoclaved. For use on the mobile units a piece of gauze is interposed between the bottom and top crystal dish so as to avoid breakage of these dishes in transit.

The needle holder is placed on the working stand between two bleeding tables; the weight of the needle holder is sufficient to keep it from sliding around on the table. The crystal dish cover is lifted, the chromium lid removed, and

the crystal cover replaced. In use the cover is raised with one hand and a sterile syringe containing novocain is inserted into the upright shank of the needle, which is removed without touching any of the adjacent needles, and the crystal dish cover is then replaced. A similar procedure is carried out in each individual case.

This needle holder has been in use at the Blood Donor Center in Chicago for the past four months where approximately 100,000 venepunctures have been done with a large varying personnel. It has been found to be a simple and time-saving method for changing needles. It has been satisfactory in overcoming the objection of the cork needle holders. It is our opinion that physicians performing mass immunizations or allergists performing numerous intra-dermal tests might find this type of needle holder useful. Its depth could be varied to hold needles of other lengths.



## BOOK REVIEWS

**Outline of the Amino Acids and Proteins.** Edited by *Melville Sahyun, M.A., Ph.D.*, Vice President and Director of Research, Frederick Stearns and Company, Detroit, Mich., and thirteen contributing authors. Reinhold Publishing Corporation, New York, N. Y. Price \$4.00. Cloth with 260 pages, illustrated.

It is now generally appreciated that amino acids and proteins play both a unique and essential role in life processes. In planning the make-up of the book, the author takes the commendable position that if one is to understand the manifold functions of amino acids and proteins, a knowledge of their occurrence, composition, physical chemical properties, and behavior is essential. The subject matter is presented in twelve short and concise chapters. The first six chapters deal with the discovery of the amino acids; the occurrence, amino acid content, and properties of proteins; the structure of the protein molecule and the hydrolysis of proteins; the synthesis and isolation of certain amino acids; and the methods of analysis for amino acids and proteins. The last six chapters consider the relation of amino acids and their derivatives to immunity; the relation of amino acids to biologically important products and the role of certain amino acids in detoxication; the metabolism of proteins and amino acids and the intermediary metabolism of individual amino acids; the significance of amino acids and proteins in nutrition; and nitrogen equilibrium and the biological value of proteins.

It is appreciated that in recent years several comprehensive texts dealing with the chemistry of the amino acids and proteins have appeared. The present book does not aim to cover all phases of the subject nor does it attempt to treat any subject exhaustively. It is written with the purpose of outlining in a simple and readable manner the essentials of the chemistry and biochemistry of the amino acids and proteins and to serve as a background for those who wish to go into the subject more deeply. In this respect the book would appear to have a definite place and should be well received by students and teachers of biochemistry as well as by clinicians desiring readable information on the subject.

E. M.

**Manual of Clinical Mycology.** Prepared under the auspices of the Division of Medical Sciences of the National Research Council. By *Norman F. Conant, Ph.D.*, Assistant Professor of Bacteriology, Duke University School of Medicine, and Mycologist to Duke Hospital; *Donald Stover Martin, M.D.*, Associate Professor of Bacteriology and Associate in Medicine, Duke University School of Medicine; *David Tillerson Smith, M.D.*, Professor of Bacteriology and Associate Professor of Medicine, Duke University School of

Medicine; *Roger Denio Baker*, M.D., Associate Professor of Pathology, in charge of Surgical Pathology, Duke University School of Medicine; and *Jasper Lamar Calloway*, M.D., Assistant Professor of Medicine, in charge of Dermatology and Syphilology, Duke University School of Medicine. W. B. Saunders Co., Philadelphia and London, 1944. Price \$3.50. Cloth, 5¼ by 7¾ inches with 348 pages and 148 figures.

This book follows the style of other military medical manuals published by the Saunders Company particularly for the Armed Forces. It is small, compact, well illustrated, well printed, and has few typographical errors. The 148 figures make up 11 world maps which show the distribution of some of the mycoses; 19 roentgenographs; 37 illustrations; and 197 photographs of mycologic, clinical, and pathologic aspects of the diseases.

Each of the five authors was responsible for certain sections which were incorporated in each chapter and the whole rewritten and coordinated by Donald S. Martin. Norman F. Conant contributed the mycology section; Donald S. Martin, the section on geographic distribution and immunology; David T. Smith prepared the symptomatology, differential diagnosis, prognosis, and treatment of the systemic diseases; Roger D. Baker was responsible for the pathology; and Jasper L. Calloway contributed the clinical aspects of the dermatomycoses and other superficial mycoses. There are twenty-five chapters, the last two dealing with the fundamentals of elementary mycology and contaminants. The appendix contains a formulary and deals with methods of study. The index is complete.

The recognition of the importance of mycotic infections is borne out by the amazing increase in publications of case reports, experimental investigations, and, more recently, textbooks. The controversies as to the nomenclature of fungi, however, still exist. In this respect, the authors have tried to simplify the identification and classification of pathogenic fungi. In so doing, however, many of the commonly accepted names have been eliminated or reduced to synonymy with other lesser known names with the result that more confusion will exist. Examples of this are *Trichophyton purpureum*, which is referred to as *T. rubrum*, and *Achorion Schönleini*, which is changed to *Trichophyton Schönleini*. *Paracoccidioides brasiliensis*, *P. cerebriformis*, and *P. tenuis*, the agents of South American blastomycosis, are all transferred to the incorrect, but still maintained genus *Blastomyces* as the single species *B. brasiliensis*. The genus *Actinomyces* is used only for the anaerobic, gram-positive and non-acid-fast organisms represented by the single species, *A. bovis*, whereas the aerobic forms which are also Gram-positive and some of which are acid-fast are called *Nocardia*. Such a separation can be very confusing, especially in view of the work of some investigators who find that oxygen requirements can be reversible with this group of organisms.

To simplify the identification of fungi in the laboratory, the authors recommend the use of blood agar incubated at 37° C. Unfortunately, such a procedure, if at all useful, can be so only in the hands of a trained mycologist and should not be relied upon for species identification.

The clinical discussions for most of the systemic mycoses are well presented, although brief for some diseases. In contrast, the methods of treatment for

some of the mycoses are numerous and might be confusing to the clinician. The use of vaccine desensitization is stressed, especially in certain patients with blastomycosis. The known immunologic phenomena are well presented although the value of skin testing with fungous extracts in many mycoses is considered either as doubtful or nonspecific. The sections on pathology emphasize particularly the appearance of the organism in tissue but also present most of the salient features of the pathologic processes. Unfortunately, not all of the diseases described have sections on pathology.

This book should be a valuable addition to one's library. It is easy to handle, inexpensive, and contains recent information both for the clinician and for the laboratory technician.

**Etiology, Diagnosis and Treatment of Amebiasis.** By *Charles F. Craig*, M.D., M.A. (Hon.), F.A.C.S., F.A.C.P., Colonel, U.S.A., Retired, D.S.M.; Late Commandant, Army Medical School, and Assistant Commandant, Army Medical Center, Washington, D.C.; Emeritus Professor of Tropical Medicine, Medical School, Tulane University of Louisiana, New Orleans, La. Williams & Wilkins Company, Baltimore, 1944. Price \$4.50. Cloth with 332 pages.

This is a priceless encyclopedia on the important subject of Amebiasis written by the world's renowned authority. Every phase of the subject is systematically presented with the usual clearness and conciseness which characterize Colonel Craig's writings. It contains all recent and valuable information accumulated in numerous researches since the first publication of the author's treatise on *Amebiasis and Amebic Dysentery* in 1934. These additions, together with those preceding, which have undergone the author's careful and painstaking scrutiny, based on his wide personal experience, are the highlights of the book.

We may say that Amebiasis would seem sufficient for the title of the book without specialization, as every phase of the subject is included.

The book is strongly recommended to all those engaged in clinical and laboratory medicine.

H. T.

**Aids to Clinical Pathology Including Postmortem Technique.** By *David Haler*, M.B., B.S. (Hons.) London, D.C.P. London, Hon. Pathologist, All Saints' Hospital, London, and Pathologist, Emergency Medical Service; Member of L.C.C. Panel of Special Pathologists; Williams & Wilkins Co., Baltimore, 1944. Cloth with 358 pages.

**The Reticulo-Endothelial System in Sulfonamide Activity.** By *Frank Thomas Maher*, Ph.D., Assistant Professor of Pharmacognosy and Pharmacology. University of Illinois Press, Urbana, Ill. Price \$2.50. Paper bound with 232 pages and 23 figures.

# THE QUANTITATIVE DETERMINATION OF THE SERUM BILIRUBIN WITH SPECIAL REFERENCE TO THE PROMPT-REACTING AND THE CHLOROFORM-SOLUBLE TYPES

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SINCE the original description by van den Bergh and Snapper<sup>1</sup> of a method for the quantitative estimation of bilirubin, based on the Ehrlich-Proscher diazo reaction, many modifications have been advocated.<sup>2-10, 22</sup> All of these have represented an attempt to avoid the three main sources of error inherent in the original method: namely, (1) loss of bilirubin by adsorption on the protein precipitate; (2) variations in the azobilirubin color due to changes in pH; and (3) imperfect color match with the standard color solution originally recommended.

Regarding the qualitative aspects of the color reaction, van den Bergh and Müller,<sup>11</sup> in 1916, first noticed the differing behavior of the "direct"- and "indirect"-reacting serum bilirubins, which has subsequently become so well known. In 1934 van den Bergh and Grottepass<sup>7</sup> described a colorimetric method for the quantitative evaluation of direct and indirect fractions of the serum bilirubin. This publication likewise, was followed by a number of proposed modifications.<sup>9, 12-14</sup>

Of all of the methods which have been proposed, the one described by Malloy and Evelyn<sup>9</sup> has met the most general acceptance, since it permits an accurate estimation of total bilirubin and a ready distinction between the direct- and indirect-reacting fractions. Unfortunately, Malloy and Evelyn recommend reading the total direct-reacting bilirubin only after 30 minutes as a routine procedure, and for a more detailed study of the behavior of the direct reaction they recommend readings at 10, 30, 60, and 120 minutes. As will be emphasized in detail later, the 10-minute reading already combines the prompt and delayed types, which have entirely different significance, since the available evidence indicates that the delayed direct- and the indirect-reacting types are much more nearly related and that the delayed type should, accordingly, be estimated separately. Even the 5-minute reading as proposed by Cantarow and his associates<sup>15</sup> has the same disadvantage.

The time limit for the prompt as compared with the delayed is bound to be somewhat arbitrary. Based upon studies of bile bilirubin and sera from cases of mechanical jaundice, on the one hand, as contrasted with hemolytic

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jaundice sera or hemorrhagic bilirubin containing fluids from body cavities on the other,<sup>1b</sup> it appears certain that nearly all of the prompt-reacting type give rise to azobilirubin formation within 1 minute, while the delayed direct-reacting type has not yet begun to produce color at this time. Admitting that there may be slight overlapping in some instances, it is nevertheless certain that a division at 1 minute offers the greatest likelihood of a sharp separation of the two forms when the basis of separation is speed of reaction only.

The separate measurement of the direct- and indirect-reacting bilirubins has been approached from a different angle following the first report and subsequent papers of Grunenberg<sup>16-18</sup> who noticed that the indirect-reacting bilirubin is soluble in chloroform, while the direct-reacting type is not. His observations have been confirmed by a number of investigators, and some of them have used this differing behavior for the quantitative estimation of both types of bilirubin.<sup>19-21</sup> Heilbrun and Hubbard<sup>22</sup> have applied the chloroform solubility of the indirect-reacting bilirubin to perform an improved ieteric index. No mention has been made of the question of chloroform solubility of the delayed direct-reacting bilirubin.

The description by Feigl and Querner<sup>23</sup> of the so-called biphasic reaction complicated the interpretation of the qualitative van den Bergh. McNee<sup>24</sup> believes that this type of reaction is due to the presence in the serum of certain proportions of both direct- and indirect-reacting bilirubins. We believe the biphasic reaction is better explained by the relative amount present of prompt-reacting and delayed direct-reacting bilirubins. As noted, however, the latter is believed to have the same significance as the indirect-reacting bilirubin. Hence, it is important to differentiate both types of direct-reacting bilirubins in any quantitative method. This is easily achieved by a slight modification of the Malloy and Evelyn technique consisting simply of making the reading for the prompt direct-reacting bilirubin at 1 minute after the addition of the reagent to the diluted serum.<sup>25</sup> The total direct-reacting bilirubin is read at 15 minutes. In some sera with a high content of the delayed direct-reacting bilirubin some of it is unable to react within 15 minutes, but this is of little moment since any that does not react directly will be picked up in the total reaction following addition of alcohol. The 15-minute period is purely arbitrary, since the longer one waits, the more of the so-called indirect-reacting bilirubin will react directly, that is, without addition of alcohol. Actually it may be superfluous to measure the delayed or 15-minute value at all, and we believe that this can be omitted without seriously affecting the information derived, either from a clinical or fundamental standpoint. We have, however, retained the 15-minute delayed reading for purposes of comparison, as given below, and we feel that it has some value in serving as a dividing zone between the pure prompt type reacting within 1 minute and the pure indirect which has not reacted after 15 minutes except with the aid of alcohol. In other words, the 15-minute value permits a sharper separation of the prompt-reacting and the indirect-reacting types. The reading for the total bilirubin is made 15 minutes after the addition of alcohol.\*

To recapitulate:

1. The 1-minute reading: prompt direct-reacting bilirubin.

\*Malloy and Evelyn allowed a 30-minute period after addition of alcohol. Our experience has shown that the values are the same after 15 minutes as after 30 minutes.

2. The 15-minute reading: total direct-reacting bilirubin.
3. The difference between the 1- and 15-minute reading: delayed direct-reacting bilirubin.
4. The 15-minute reading after adding alcohol: total bilirubin.
5. The difference between this and the reading for total direct-reacting bilirubin: indirect bilirubin.

Cantarow and co-workers,<sup>26</sup> on the basis of their study of the direct (30-minute)-reacting bilirubin, comment: "It is obvious that the production of the qualitative direct reaction, at least in serums of relatively low bilirubin content, is not determined by the concentration of direct-reacting bilirubin or by the proportion which it constitutes of the total. It must be remembered, however, that readings of the qualitative reactions are made at 30 minutes, which may be responsible, in part at least, for the discrepancy." These investigators found higher values for direct-reacting bilirubin (30 minutes) in sera from patients with cirrhosis or mild degrees of hepatitis than in the controls, both groups having a normal concentration of total serum bilirubin. These results have not been confirmed by others.<sup>27</sup> Measuring the prompt (1-minute) bilirubin, Delgado<sup>28</sup> found that the type of qualitative van den Bergh reaction depends upon the amount of prompt bilirubin present as can be seen in Table I.

TABLE I

CORRELATION BETWEEN THE AMOUNT OF DIRECT-REACTING BILIRUBIN AND THE TYPES OF THE QUALITATIVE VAN DEN BERGH REACTION (DELGADO<sup>28</sup>)

NUMBER OF DETERMINATIONS	QUALITATIVE VAN DEN BERGH	PROMPT BILIRUBIN (1 MINUTE) RANGE (MG. PER 100 C.C.)	TOTAL BILIRUBIN RANGE
50	Indirect	0.00 to 0.15	0.70 to 16.04
16	Delayed biphasic	0.15 to 0.88	0.63 to 3.27
40	Prompt biphasic	1.01 to 6.98	2.60 to 17.18

Sepúlveda and Osterberg<sup>29</sup> have recently applied the method of Varela-Fuentes and Recarte<sup>20</sup> to the photometer (Sheard-Sanford) in order to measure the chloroform-soluble bilirubin. The values for direct-reacting bilirubin are obtained in dilutions according to the Malloy-Evelyn method, but this is determined only when the qualitative van den Bergh reaction is prompt or biphasic, and even then the time of the determination is rather indefinite, being "made during the first 10 minutes after alcohol is added."<sup>29</sup> The evident purpose of this is to avoid the error due to slow coupling of the indirect-reacting bilirubin with the diazo reagent even in aqueous solution. It has appeared to us that the use of the 1-minute reading with the Malloy-Evelyn method, as already referred to, makes procedures of the Sepúlveda-Osterberg type quite superfluous, since the former is much simpler and is not liable to variables which may not be recognized, such as loss incident to chloroform extraction, emulsion, heating, and other factors.

The purpose of the present investigation, therefore, has been a comparison of the described modification of the Malloy-Evelyn method with the Sepúlveda-Osterberg procedure. An additional purpose was to study the criticisms of the Malloy-Evelyn method recently advanced by With.<sup>30</sup>

## EXPERIMENTAL

The Malloy-Evelyn method, with the time modifications noted in the foregoing, was compared with the Sepúlveda-Osterberg method on forty-one human blood sera of varying bilirubin content. All readings were made with an Evelyn photoelectric colorimeter, calibrated in the usual manner with crystalline bilirubin (see Malloy and Evelyn<sup>9</sup>). The center setting for the Sepúlveda-Osterberg modification was obtained with the same mixture of chloroform-alcohol which they recommend for redissolving the dry residue after the chloroform extraction rather than with water as suggested by them. As will be seen in Table II, the values for the chloroform-soluble bilirubin obtained with their method were, for the most part, lower than those obtained by calculating the difference between the total bilirubin and the total direct (15-minute) value and were, of course, much lower than the difference between prompt and total.

TABLE II  
COMPARISON BETWEEN SEPÚLVEDA-OSTERBERG AND MALLOY-EVELYN PROCEDURES  
(VALUES IN MG. PER 100 C.C. OF SERUM)

SERUM	SEPÚLVEDA- OSTERBERG (CHCl <sub>3</sub> SOL.)	1 MIN.	PER CENT OF TOTAL	15 MIN.	PER CENT OF TOTAL	TOTAL	INDIRECT
1	0.33	0.74	34.2	1.48	68.5	2.16	0.68
2	0.44	2.96	72.0	3.50	85.3	4.10	0.60
3	1.00	5.20	63.8	6.80	83.5	8.14	1.34
4	1.30	4.28	57.3	6.12	82.0	7.46	1.34
5	0.99	0.23	15.3	0.62	41.3	1.50	0.88
6	1.01	8.28	46.8	10.76	60.8	17.68	6.92
7	0.72	0.62	40.0	1.23	79.3	1.55	0.66
8	0.49	3.94	50.0	5.58	70.8	7.88	2.30
9	2.40	9.90	52.8	13.40	71.5	18.74	5.34
10	0.09	0.12	19.0	0.32	50.8	0.63	0.31
11	9.90	0.76	5.0	1.60	12.1	13.20	11.10
12	0.20	0.86	35.8	1.60	66.6	2.40	0.80
13	1.70	4.38	47.2	6.60	71.1	9.28	2.68
14	0.40	0.67	54.0	0.96	77.4	1.24	0.28
15	0.87	2.00	50.5	2.96	74.7	3.96	1.00
16	0.86	2.28	31.2	4.60	63.0	7.30	2.70
17	2.90	6.54	28.0	13.20	57.9	22.80	9.60
18	1.80	0.50	13.8	0.90	25.0	3.60	2.70
19	2.10	0.57	19.7	1.37	47.5	2.88	1.51
20	0.88	4.46	69.6	4.82	75.3	6.40	1.58
21	1.10	4.16	57.6	5.58	77.2	7.22	1.64
22	0.76	3.44	57.3	4.68	78.0	6.00	1.32
23	1.09	5.39	60.3	7.08	79.1	8.94	1.86
24	0.40	1.47	40.8	1.87	51.9	3.60	1.73
25	0.95	0.28	16.1	0.67	38.7	1.73	1.06
26	0.51	3.82	65.8	4.60	79.3	5.80	1.20
27	0.47	0.38	33.3	0.81	71.0	1.14	0.33
28	0.40	5.20	57.7	7.46	82.8	9.00	1.54
29	2.51	13.94	56.2	17.28	69.6	24.80	7.52
30	0.07	0.55	50.0	0.77	70.0	1.10	0.33
31	1.31	5.44	46.8	7.53	64.8	11.60	4.08
32	0.24	0.11	22.0	0.22	44.0	0.50	0.28
33	0.72	1.27	55.2	1.32	57.3	2.30	0.98
34	0.27	4.02	46.9	6.38	74.5	8.56	2.18
35	0.83	2.46	47.3	3.78	72.6	5.20	1.42
36	0.36	1.20	50.0	1.86	77.5	2.40	0.54
37	1.33	11.36	61.0	14.72	79.1	18.60	3.78
38	3.00	0.40	8.8	0.90	20.0	4.50	3.60
39	0.20	0.24	31.6	0.38	50.0	0.76	0.38
40	0.72	2.01	47.8	2.76	65.7	4.20	1.44
41	4.20	0.83	11.2	1.24	16.8	7.38	6.14

The question arose as to the efficiency of extraction of the indirect-reacting bilirubin by the chloroform. This was studied in the following way: 1 c.c. portions of various sera were extracted exhaustively with chloroform; this could be ascertained because the chloroform became colorless and the dry residue of the last extract when redissolved in chloroform and alcohol gave a negative van den Bergh reaction. The supernatant fluid after the chloroform extraction was then treated according to the modified Malloy-Evelyn technique and, as noted in Table III, an important fraction of the indirect-reacting bilirubin was still present; in other words, the chloroform extraction was not particularly efficient.

TABLE III

VALUES FOR PROMPT AND DELAYED DIRECT REACTING AND TOTAL BILIRUBINS IN THE SUPERNATANT FLUID OF SERA EXTRACTED EXHAUSTIVELY WITH CHLOROFORM

SERUM	1-MIN. VALUE IN MG.	15 MIN. VALUE IN MG.	TOTAL IN MG.
	PER 100 C.C.	PER 100 C.C.	PER 100 C.C.
a	2.12	5.08	4.24
b	0.17	0.32	0.42
c	1.08	2.01	3.00
d	1.65	1.87	2.43
e	0.99	1.27	2.03
f	0.37	0.75	1.5
g	0.18	0.47	0.85
h	2.59	1.72	4.02
i	4.02	7.73	9.25
j	0.52	0.91	3.6

With<sup>20</sup> has recently stated that the Malloy Evelyn method gives low values because the excessive dilution slows the reaction. He compared this method with Jendrassik and Grof's procedure<sup>10</sup> but added alkali (Fehling II) in both instances; he concluded that results obtained with the Malloy-Evelyn method are from 25 to 50 per cent lower. This was explained as being due to a weaker catalytic activity on the part of methyl alcohol than of the caffeine-sodium benzoate used by Jendrassik and Grof.

In order to study this point, the values for total bilirubin were obtained in four sera using the Malloy-Evelyn procedure and also using caffeine-sodium benzoate instead of alcohol. Readings were first made without addition of alkali, after which 1 c.c. of the Fehling II solution was added to each tube and readings were again taken, this time with a 520 filter rather than the 540 filter as regularly used. This change is also in accordance with the Jendrassik-Grof method. The data are given in Table IV where it is seen that the values obtained when methyl alcohol was used are uniformly higher than those when caffeine-

TABLE IV

VALUES FOR TOTAL BILIRUBIN USING METHYL ALCOHOL AND CAFFEINE-SODIUM BENZOATE WITH AND WITHOUT ADDITION OF ALKALI

SERUM	BILIRUBIN IN MG. PER 100 C.C. (WITHOUT ADDING ALKALI)		VALUES IN EVELYN READINGS* (ADDING ALKALI)	
	METHYL ALCOHOL	CAFF.-SOD. BENZ.	METHYL ALCOHOL	CAFF.-SOD. BENZ.
a	2.88	1.31	96	802
b	6.48	2.24	92	83
c	7.30	3.51	921	833
d	6.00	2.30	913	81

\*No calibration was made so that the Evelyn readings are given directly. It must be borne in mind that the higher the reading, the lower the concentration.



sodium benzoate was used, but when alkalinized the reverse is noted. From this it is clear that it is not a matter of differing catalytic effect but rather an effect of the alkali and caffeine on the color intensity of the azobilirubin. This undoubtedly explains the lower values obtained by With for the Malloy-Evelyn procedure when alkali is added. It was not recommended by Malloy and Evelyn, nor was it used in the present study.

#### DISCUSSION

Analysis of the present results shows that the chloroform-soluble bilirubin as determined by the Sepúlveda-Osterberg method is regularly less in amount than the indirect bilirubin fraction determined by the Malloy-Evelyn procedure. This is probably due to incomplete extraction by the chloroform, evidence for which is presented.

Varela-Fuentes and his associates<sup>31</sup> believe that the extraction of the chloroform-soluble bilirubin is incomplete but that no essential difference exists between the chloroform-soluble fraction and the indirect-reacting bilirubin. Lopez-García and Zelasco<sup>21</sup> extract but once with chloroform, employing this extract in a semiquantitative fashion and recognizing that chloroform extraction is incomplete and that the material extracted may not be an entity.

The quantitative difference between the Sepúlveda-Osterberg method and that of Malloy-Evelyn is more striking if it is taken into consideration that the amount of indirect bilirubin (Malloy-Evelyn) has been calculated from the difference between the total direct-reacting and the total bilirubins. As the delayed direct-reacting and indirect-reacting bilirubins are believed to have the same significance, the important difference from a clinical standpoint is that between the prompt direct-reacting bilirubin and the total. This, of course, gives still larger differences, as noted in Table II. According to the present results, the chloroform extraction method is not reliable as a quantitative procedure, and since it is, in addition, much more cumbersome and time-consuming than the Malloy-Evelyn method, it is not recommended.

The accepted end point for the qualitative prompt direct van den Bergh reaction is 1 minute. This is based upon van den Bergh's original observations on various icteric sera, fluids, and bile. If a quantitative expression of this reaction is desired, it is logical to obtain the first reading at the end of 1 minute, that is to say, to read the prompt direct-reacting bilirubin. The delayed direct-reacting fraction is much more arbitrary in that the longer one waits, the more of the indirect-reacting bilirubin will react directly, that is to say, without alcohol or other catalytic agent. This phenomenon has been observed repeatedly, especially with hemolytic jaundice sera. In general, it is probably sufficient to make use of but two readings; the 1-minute, or prompt, and the total, following addition of alcohol. The 15-minute direct reading may be reserved as a means of separating more sharply the prompt and the indirect fractions. It is quite possible that insufficient attention has been given, in clinical diagnosis, to the ratio between prompt and total bilirubins.

The percentage that the prompt bilirubin represents of the total pigment (Table II) is not at all proportional to the percentage which the total direct-reacting bilirubin represents of the total; this explains the lack of relationship

found by some investigators<sup>20</sup> between the qualitative van den Bergh and the percentage of direct bilirubin.

## SUMMARY AND CONCLUSIONS

1. A slight modification of the Malloy-Evelyn technique for serum bilirubin is recommended, which facilitates estimation of the prompt- and delayed direct-reacting types as well as the total bilirubin. The values obtained at 1 minute represent a quantitative expression of the qualitative prompt direct van den Bergh reaction.

2. The bilirubin extracted from the serum by chloroform, according to the method of Sepúlveda-Osterberg, is not equivalent either to the indirect-reacting or the delayed direct-reacting bilirubins. In the majority of instances it is less than either, and in all instances much less than the sum of the two. The lower values obtained by chloroform extraction are due to incomplete extraction. Since the delayed direct and the indirect fractions are believed to have identical significance, chloroform extraction is held to be inadequate and superfluous.

3. The objection of With to the Malloy-Evelyn method, based on the assumption that methyl alcohol has a weaker catalytic activity than caffeine-sodium benzoate, has been found to be valid only for the alkaline solution employed by With and not for the solution prepared by the Malloy-Evelyn technique and used in the present study.

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## THE PATHOLOGY OF EXPERIMENTAL CHOLINE DEFICIENCY IN DOGS

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**S**TUDIES on the experimental production of acute choline deficiency in young puppies and of liver function in these animals have been reported by McKibbin, Thayer, and Stare.<sup>1</sup> Standard liver function tests were used, and the results of the tests were correlated with the lipid content of the livers. The present study is concerned with the lesions in these dogs and the correlation of morphologic changes with abnormal liver function tests

### METHOD

Litters of weanling puppies were used in these experiments, and the choline deficient diet was started after a preliminary observation period of ten days. The diets and the liver function tests have been described in detail by McKibbin and co-workers.<sup>1</sup> The chief source of protein was peanut meal, which was used because of its low methionine content.

At the end of the experiment, the puppies were killed by intravenous injection of sodium pentobarbital immediately after the last series of liver function tests had been performed.\* Tissues were fixed in 10 per cent formalin solution, and the stains used were hematoxylin-eosin, Sudan IV, and Ziehl-Neelsen (for ceroid<sup>2</sup>).

### RESULTS

Data concerning the choline supplementation of the diet, the weights of the liver and kidneys, liver and body weight ratio, liver lipid content, and the results of the various liver function tests are given in Table I. At the time of autopsy, all of the animals which had been fed rations deficient in choline were undernourished. The tendency of the blood to clot was not so evident in deficient as in control animals, and marked pallor of the viscera (anemia) was noted in two of the deficient puppies (Dogs 31 and 32). The livers of the deficient animals were pale yellow or pinkish yellow. The thymuses of the animals which had received no supplements of choline or methionine were invariably smaller than those of littermate controls. In many instances there was no recognizable thymic tissue. When such tissue was present, there was no gross alteration other than the small size of the organ. In Dog. 32 there was edema of the stroma of the thymus.

The microscopic examination revealed consistent abnormalities of the livers and thymuses of the dogs which had been fed diets deficient in choline. The livers of those which had received no supplemental choline or methionine con-

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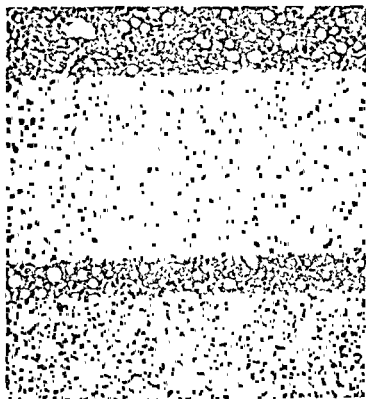
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\*Dogs 1 to 11 and 24 to 30 are not included in this study because these early diets were deficient in factors other than choline, as indicated by suboptimal growth and abnormalities in the control animals.

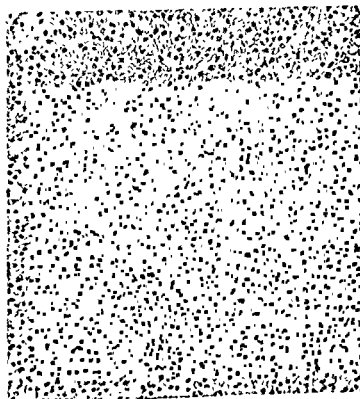
TABLE I  
DEFICIENCY OF CHOLINE IN DOGS

LIT- TER	RA- TION	DOG	SUPPLE- MENT	DURATION OF EXPER. (DAYS)	PER CENT WEIGHT CHANGE DURING EXPER.	LIVER WEIGHT (GM.)	LIVER WEIGHT BODY WEIGHT × 100	LIVER LIPIDS (CHLORO- FORM- EXTRACT- ABLE) (PER CENT)	KIDNEY WEIGHT (TOTAL)	BROMSUL- FALEIN TEST (μG. DYE PER ML. PLASMA)	PLASMA PHOS- PHATASE (μG. P LIBERATED)	PRO- THROM- BIN TIME (SEC.)	PLASMA TOTAL CHOLE- STEROL (MG./100 C.C.)	PLASMA CHOLE- STEROL ESTERS (MG./100 C.C.)
III	3	12	None	43	- 3.0	155	6.1	50.5	28	12	397	998	23.0	
		13	None	43	- 10.4	130	6.0	48.1	22	25	624	1,230	13.0	
		14	150 mg. %	43	+ 49.0	230	7.7	7.4	28	4	122	260	8.0	
		15	150 mg. %	57	+182.0	205	5.5	13.3	27.5	3	79	231	10.8	
		16	150 mg. %	57	+254.0	225	4.2	16.0	43.4	5	87	222	10.8	
		17	None	32	+ 12.0	120	6.0	47.1	21	31	563	1,080	62.0	
IV	3	18	None	32	- 8.5	90	4.3	56.6	18	42	566	1,183	46.0	
		19	None	32	+ 4.9	115	3.8	48.2	25	22	614	1,124	26.0	
		20	0.7% dl- methionine	32	+ 57.0	170	5.0	11.9	29	10	82	245	13.7	
		21	150 mg. %	32	+ 24.6	140	4.9	19.1	24	4	137	349	10.5	
		22	150 mg. %	32	+ 43.2	160	4.5	14.1	27	4	112	300	12.0	
		23	150 mg. %	32	+ 30.1	140	5.9	12.3	23	5	143	399	8.8	
VI	5	31	None	18	- 6.7	129	7.7	34.6	28	25	1,423	1,423	55.0	9
		32	None	21	+ 9.3	105	7.2	46.3	29	27	1,403	1,403	23.8	22
		33	10 mg. %	60	+ 71.0	168	4.85	48.7	44	20	629	1,338	16.3	37
		34	25 mg. %	60	+ 86.9	184	5.4	43.2	-	20	562	1,098	16.2	27
		35	50 mg. %	60	+335.0	235	4.4	25.9	46	9	114	316	11.8	58
		36	100 mg. %	60	+441.0	317	4.15	16.9	62	9	72	239	12.9	95

tained much lipid material in vacuoles. The vacuoles varied from small to large, the latter being more numerous (Fig. 1, A). Supplemental choline chloride in amounts of 10 mg. per 100 Gm. of ration (Dog 33) did not prevent the presence of fat similar in quantity and appearance to that of animals which had received no supplemental choline. One animal which received 25 mg. of choline chloride per 100 Gm. of ration (Dog 34) had appreciably less fat in its liver, and one which received 50 mg. per 100 Gm. of ration (Dog 35) had a liver in which there



A.



B.

Fig. 1.—A, Diffuse fatty metamorphosis of liver of Dog 31. (No supplemental choline.)  
B, Focal fatty metamorphosis in Dog 35 (50 mg. choline per 100 Gm. ration). (Hematoxylin and Eosin,  $\times 150$ .)

were only small numbers of cells which contained visible fat vacuoles (Fig. 1, B). The livers of animals which received suboptimal amounts of choline had relatively few cells which contained large fat vacuoles, but there were minute vacuoles of visible fat in nearly all cells. The large vacuoles were in focal areas in the lobules and were central in some and peripheral in others. One hundred milligrams of choline chloride per 100 Gm. of ration protected the liver against fatty metamorphosis. Equal protection was afforded in the absence of choline by 0.7 Gm. of dl-methionine per 100 Gm. of ration (Dog 20). The livers of several deficient dogs were stained by acid-fast technique for ceroid, and none of this substance was demonstrated.

The thymuses of the puppies fed diets deficient in choline were absent in most instances. Those which were found and examined microscopically differed from the thymuses of the control animals. There were few thymocytes in the medullary portions of the glands of the deficient animals, and the stroma was therefore prominent. The cortices were relatively thin, and the thymocytes were not so densely packed together as in the control animals. The interlobular connective tissue was more prominent in the deficient animals and appeared edematous. The most striking change was in the thymic corpuscles. In the control animals, these were relatively small, and the cells comprising them had homogeneous pink cytoplasm and reticulated nuclei. In the deficient animals, they were represented by large cells often multinucleated. The cytoplasm stained dark pink and vacuoles were frequent. Many had basophilic granular debris within the cytoplasm. The nuclei varied considerably, some being pyknotic and lying in lacunae in the cytoplasm, others being large and pale with aggregations of dark chromatin in focal collections within them. Some had small clear intranuclear vacuoles.

Microscopic examination of brain, heart, lung, spleen, pancreas, adrenal, kidney, testis, ovary, stomach, intestine, eye, and costochondral junction of the choline-deficient puppies revealed no abnormalities.

#### DISCUSSION

The lesions of the puppies which had been fed choline-deficient rations were not observed in control animals fed similar rations and supplemental choline or methionine. The fact that optimal growth was not obtained from diets which were adequate in all substances but choline is apparent. The cause of the anemia noted in two animals is not known, but presumably it was related to choline deficiency since none of the control dogs was significantly anemic. The anemic dogs were the only ones which had received Ration 5 without supplemental choline.

Although the fatty livers of choline deficiency have heretofore been ascribed to fat infiltration, the irregular distribution of the fat in the livers of the puppies of our series is unlike that of pure fat infiltration in which the fat is characteristically in the periphery of the lobules. In the livers of animals receiving suboptimal amounts of choline, there were invariably some cells which contained large fat vacuoles, but Sudan stains of these livers revealed minute droplets in practically all cells even though fat was not visible in sections stained with hematoxylin and eosin. It seems probable that the lesion of the liver is a com-

bination of both fatty degeneration and fat infiltration. In the livers of animals suffering from marked deficiency, the infiltrated fat may mask that which signifies degeneration.

The diminution of liver function demonstrated by clinical tests (Table I) confirms the anatomic evidence of degeneration. It is unlikely that simple fat infiltration could be responsible for such changes in an organ known to have a factor of safety of approximately 80 per cent. Unpublished experiments from this laboratory on pantothenic acid-deficient puppies reveal little functional abnormality of the liver even though 50 per cent of its dry weight is lipid material. Thus it seems improbable that the mere presence of fat is responsible for diminution of liver function, so that while fatty metamorphosis appears to be an anatomic manifestation of cell injury in choline deficiency, it may only be coincidental.

There were variations in the levels of plasma phosphatase, cholesterol, cholesterol esters, and in bromsulfalein elimination which correlated closely with the morphologic alterations and lipid contents of the livers of the deficient puppies. The prothrombin times of the control animals were uniformly below fifteen seconds, and only one of the deficient dogs had a prothrombin time of less than fifteen seconds. In general, the dogs having large amounts of lipid in their livers had correspondingly high prothrombin times.

The ratio of liver weight to body weight varied considerably in both deficient and control dogs. There was no correlation of this ratio with the presence or absence of choline in the diet. Neither was there correlation between this ratio and the lipid content of the livers. This differs from similar observations on young rats deficient in choline. Studies on these animals have shown a remarkably constant correlation between the relative weights of the livers and the amount of lipid in the livers.<sup>3</sup>

The kidneys of the choline-deficient puppies were normal. This is in marked contrast to the kidneys of choline-deficient rats in which there are profound renal changes. These renal lesions in rats consist of epithelial necrosis of convoluted tubules and Henle's loops, together with marked engorgement of capillaries and subcapsular glomerular hemorrhages.<sup>4, 5</sup> In experiments in this laboratory, these lesions have been produced by a ration similar to the one used on the puppies.<sup>3</sup> Variations of this type are usually ascribed to differences in species, rations, or the ages of the animals used. Since the rations were comparable and the developmental ages of the deficient dogs were presumably the same or less than those of the rats at the beginning of the experiments, we believe this difference to be one of species.

The thymuses of the dogs fed choline-deficient rations were small and there was necrosis of the thymic corpuscles. There were similar changes in the thymuses of the rats which we have studied, and in some there were demonstrable stages in the disintegration of thymocytes. Because of this, the small thymuses are believed to be the result of disappearance of cells together with diminished formation of cells. The latter is indicated by almost complete absence of germinal centers. The mechanism by which deficiency of choline is responsible for these changes is unknown.



## CONCLUSIONS

The pathology of uncomplicated choline deficiency in young puppies has been described. There were fatty metamorphosis (degeneration and infiltration) of the liver and atrophic changes of the thymus. The ratio of gross liver weight to body weight did not correlate with the choline content of the diet. The kidneys, and the other tissues examined, of the choline-deficient puppies, were not morphologically abnormal.

The morphologic changes in the liver correlated with impairment in liver function and also support the growth and functional data that the choline requirement of the puppy is between 50 and 100 mg. of choline per 100 Gm. of the ration used.

Control animals fed identical rations supplemented with sufficient choline or methionine were normal in all respects.

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## RECOVERY FROM FULMINATING MENINGOCOCCIC INFECTION WITH MYOCARDITIS PROVED BY ELECTROCARDIOGRAPHY

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**M**ENINGOCOCCEMIA with meningitis and associated myocardial and pulmonary involvement is rarely seen. In 1936 Saphir<sup>1</sup> reported two cases of myocarditis with post-mortem findings. In 1939 Hartwell<sup>2</sup> reported another and was able to find a total of twelve such cases in the literature up to that time. All of these terminated fatally. We have been unable to find in the literature any record of recovery from this complication. We were also unable to find a publication which presented electrocardiographic studies in meningococcic myocarditis. The following is a case report and follow-up after six months with electrocardiographic findings of a patient who recovered from meningococcemia with meningeal, myocardial, and pulmonary involvement.

### CASE REPORT

A soldier 25 years of age was admitted to an Army hospital in the European Theater of Operations Dec. 24, 1943. He had been well until twelve hours before admission when he developed a chill followed by generalized aches, fever, and drowsiness. Physical examination on admission revealed a well-developed, well-nourished white male who responded clearly to questions. The temperature was 104.2° F. There was no rash on the skin and no petechiae were visible in the conjunctivae. The nasal mucosa and pharynx were mildly reddened. The blood pressure was 120/72; pulse rate, 128 per minute. The pupils were equal and round and reacted promptly to light. Resistance to flexion of the neck was questionable. The Kernig, Brudzinski, and Babinski signs were absent. Deep reflexes were present and equal bilaterally but the abdominal reflexes could not be elicited. On admission, blood was taken for culture which later grew about two hundred colonies of meningococci from 1 c.c. of blood. The white blood count was 27,800 with 92 per cent polymorphonuclear forms. A lumbar puncture revealed clear fluid under normal pressure with no organisms visible on direct smear. This spinal fluid contained nine erythrocytes and thirty-two leucocytes per cubic millimeter, six of the latter being polymorphonuclear forms and twenty-six lymphocytes. The total protein was 24.0 mg. per 100 c.c. and a culture of this fluid was later reported to be sterile. The patient was seen again one and one-half hours later. He complained of intense generalized pain with myalgia most marked above and below the knee joints. There was no redness, swelling, or pain within the joints. No rash was visible on the skin at this time. However, one hour later a number of petechiae were present on the trunk, and nuchal rigidity was now definite. At this time the patient appeared acutely but not critically ill. A diagnosis of meningococcemia with meningitis was made and treatment instituted at once. During the next few hours, the number of petechiae increased until all parts of the body, including the right conjunctiva, were involved. Many of the early lesions soon developed into large purpuric spots. It was evident that we were dealing with a fulminating septicemia—the so-called purpura fulminans.

The initial treatment consisted of 5.0 Gm. of sodium sulfadiazine in 100 c.c. of distilled water injected intravenously. Following this a venoclysis of 2,000 c.c. of glucose-saline solution was started. Intravenous administration of 5.0 Gm. sulfadiazine was repeated in two hours. For a period of five hours there was little change in the soldier's condition except that the blood pressure dropped to 90/60 and although of good quality, the pulse rate was 130. However, four hours later (thirteen and one-half hours after admission) the patient was found

in a state of circulatory collapse with a fast thready pulse and peripheral cyanosis; the blood pressure was unobtainable. At this time it seemed probable that the patient would die. Since no adrenal cortex extract was available, 5 minims (0.3 c.c.) of 1 to 1,000 solution of adrenalin was injected subcutaneously with a fair response. The venoclysis, which had been discontinued for fear of overburdening the cardiovascular system, was now resumed. Sodium sulfadiazine, 5.0 Gm., was added to the solution and the dose was repeated in two hours. For the next four hours the outcome remained in doubt. The systolic blood pressure ranged between 90 and 94; the diastolic pressure remained at zero for about two hours, then gradually rose to 70. A second injection of 5 minims (0.3 c.c.) of adrenalin was followed by a rise in the blood pressure to 100/70. A few hours later the pressure reached 118/72 and remained normal thereafter.

On the second day of hospitalization the condition of the patient had improved but the peripheral cyanosis persisted. The blood leucocyte count had decreased to 26,300 but the meningeal signs were quite marked. A spinal puncture released cloudy fluid under increased pressure, with 507 erythrocytes and 3,042 leucocytes per cubic millimeter; 76 per cent were polymorphonuclears and 24 per cent lymphocytes. The total protein was 114.4 mg. per cent. The direct smear revealed no organisms and the culture again was sterile. Sulfadiazine by mouth was tried but only the first dose was retained. The first blood sulfadiazine level taken about twenty hours after admission and three hours after the fourth intravenous dose of the drug was 18.6 mg. per 100 c.c. During the second twenty-four hours, ten grams were given intravenously with a rise in the blood level to 21.1 mg. per 100 c.c. free sulfadiazine. On the third day the meningeal signs and myalgia were unchanged. In addition, there was now pain and slight swelling of the right elbow as well as numbness and tingling of both feet. Most of the petechial spots had faded but the large purpuric lesions were still visible. Examination of the chest on this day (December 26) revealed signs of consolidation with bronchial breathing, bronchophony, and increased voice sounds most marked in the right mid-chest posteriorly. Crepitant râles were present over the posterior portion of the entire right side. The temperature was now 100.4° F.; pulse, 124; respirations, 40 per minute. A roentgenogram of the chest taken on a portable x-ray unit revealed extensive areas of increased density throughout both lung fields with only the left base relatively clear. These chest findings persisted for the next two days. On the following day (December 29) an x-ray picture showed marked clearing throughout the right lung and, to a large extent, also the left lung. The lungs were found to be perfectly normal in a roentgenogram taken Jan. 28, 1944. On December 27 several of the largest purpuric skin lesions showed central necrosis. The neck was less rigid with Brudzinski and Kernig signs now absent. Five days later the patient looked and felt quite well. The left foot remained swollen and painful. Weakness of the left anterior tibial muscle was noted. However, following treatment with physiotherapy, apparently normal function of this muscle was soon restored.

#### ELECTROCARDIOGRAPHIC FINDINGS

The first electrocardiogram was made December 28 (fifth day of hospitalization). This showed a regular sinus rhythm, rate 120, inverted P-waves in Lead I, S<sub>1</sub> amplitude of 1 mm., low voltage of the QRS complex, and upright T waves of low voltage in all leads. There was no elevation of the S-T segments, and the P-R interval was .17 seconds. The second electrocardiogram January 14 showed very distinct changes. There was regular sinus rhythm, rate 100, and P waves in Lead I were now upright and diphasic. The QRS complexes were of greater amplitude in all leads. T<sub>1</sub> was now upright with greater amplitude, S<sub>1</sub> 2 mm., T<sub>2</sub> diphasic, T<sub>3</sub> sharply inverted, and S-T segments not elevated in the first three leads. S<sub>4</sub> was absent, T<sub>4</sub> markedly increased in amplitude, and S-T<sub>4</sub> elevated by 2 mm. The third electrocardiogram taken January 20 showed the following differences from the previous one: There was slightly more inversion of T<sub>3</sub> and greater amplitude of T<sub>4</sub>. On January 27 the electrocardiogram showed no change from that taken one week earlier. On July 10, 1944, the electrocardio-

graphic studies revealed the following significant changes from those of Jan. 27, 1944:  $S_1$  was now absent,  $S-T_2$  elevated by 1.5 mm,  $T_2$  upright and of normal amplitude,  $T_3$  slightly diphasic but largely inverted.  $T_4$  was now 10 mm. in amplitude, 4 mm. less than previously. Except for the elevation of  $S-T_2$ , July 10, the electrocardiographic findings were normal and indicated healing of the previously noted lesion.

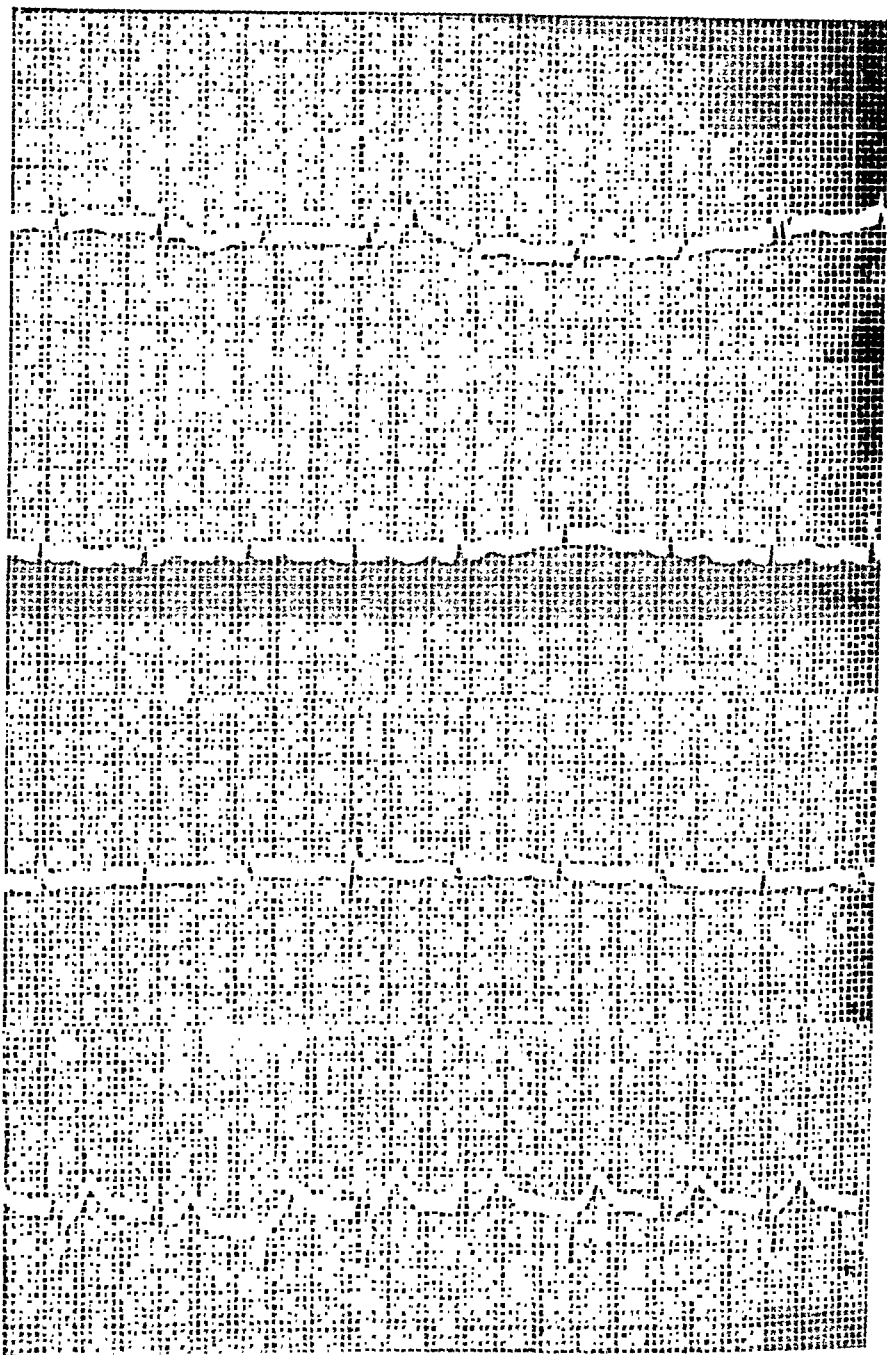
#### COURSE

The temperature on admission was 104.2° F. but following treatment dropped to 99.8° F. within twelve hours. A daily temperature of about 100° F. continued for five days, followed by a normal temperature thereafter. The pulse rate remained above 120 for the first two days with a daily rise above 100 for the first fifteen days of hospitalization. The respiratory rate on admission was 28 but rose to 42 on the third day, returning to normal four days later. The initial white blood count of 27,800 per cubic millimeter dropped to 26,300 within twenty-four hours. The count rose to 42,000 with the appearance of pneumonia on the following day. Thereafter it decreased and became normal on the eleventh day. Except for 2 plus albumin, the first urine specimen was normal. However, during the third and fourth days in the hospital, the urine was grossly bloody with a return to normal on the sixth day. Five days after the second spinal puncture the fluid was clear, under normal pressure, and contained only 6 leucocytes per cubic millimeter. Although arthralgia was present, there was no evidence of effusion at any time. By January 15 the patient wished to be out of bed but because of evidence of myocarditis in the electrocardiogram, bed rest was continued. On January 28 an x-ray of the chest revealed a heart shadow of normal size and on this day the soldier, appearing perfectly well, was transferred to another hospital for further convalescence.

Nearly six months following this transfer the soldier was seen again. He had spent about three months in a hospital convalescing from his illness. Following this he engaged in various types of light duty. At the time of this follow-up examination, however, he had engaged in moderate types of physical labor. He felt well except for slight pain in the muscles of the left leg after moderate exertion. Neurologic examination revealed some loss of epicritic sensation on the dorsum of the left foot and hyperactive deep reflexes. Physical examination was otherwise completely normal. Several of the larger purpuric spots showed superficial scarring. The cardiac response to exercise was excellent—the pulse rate being 76 at rest, increasing to 96 after exercise, and returning to 72 within thirty seconds. The blood pressure was 128/76. An x-ray picture of the chest revealed normal lung fields and a normal cardiac silhouette. The blood count, urinalysis, and sedimentation rate were normal, the last being 2 mm. in sixty minutes.

#### TREATMENT

During the first twenty-four hours a total of 20 Gm. of sodium sulfadiazine and 5,000 c.c. of glucose-saline solution were injected intravenously. It seemed evident that the danger from the fulminating infection was far greater than any toxic effect that was likely to result from large dosage of the drug. Since the drug could not be retained by mouth, 10 Gm. were given intravenously

Fig. 1. *See text for details.*

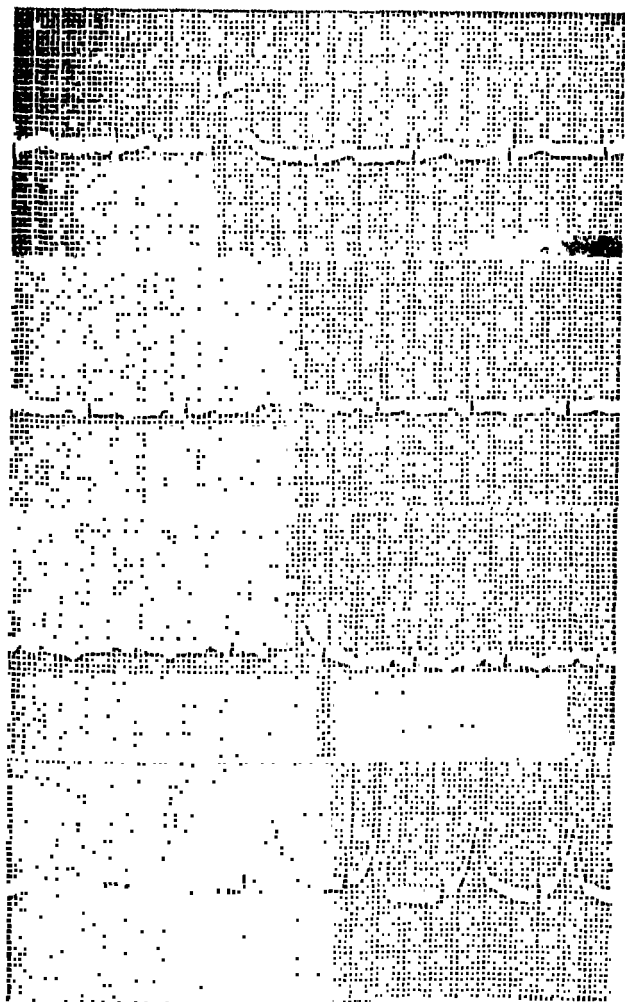


Fig. 2.—Series II taken Jan. 14, 1944

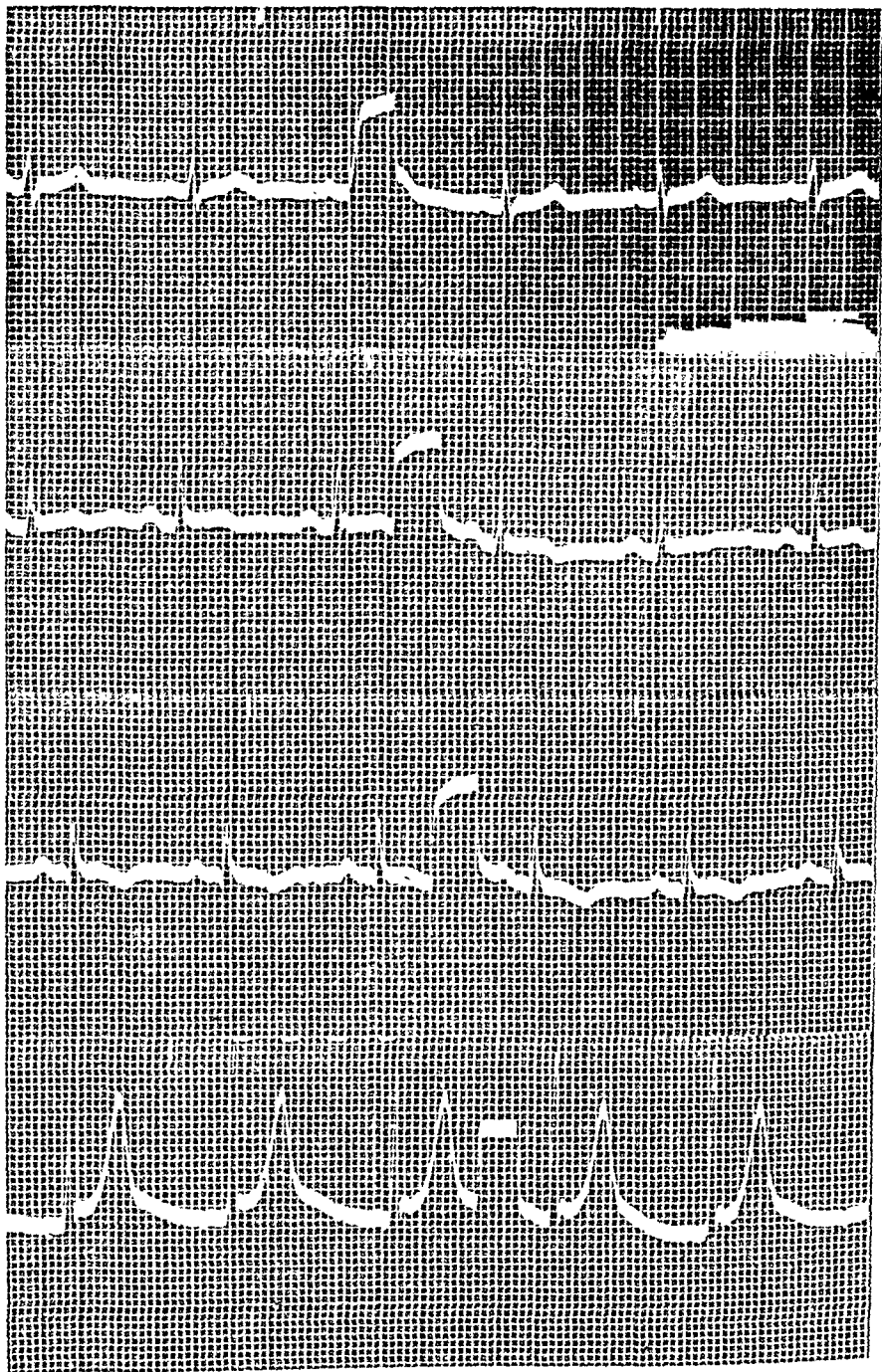


Fig. 3.—Series III taken Jan. 20, 1944.

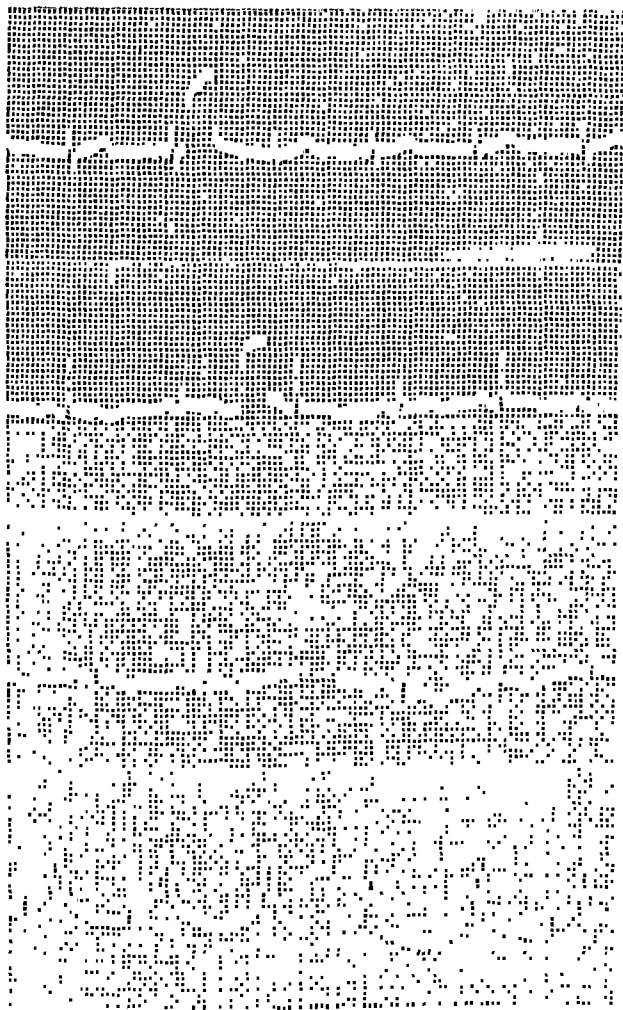


Fig. 4.—Series IV taken Jan. 27, 1944.



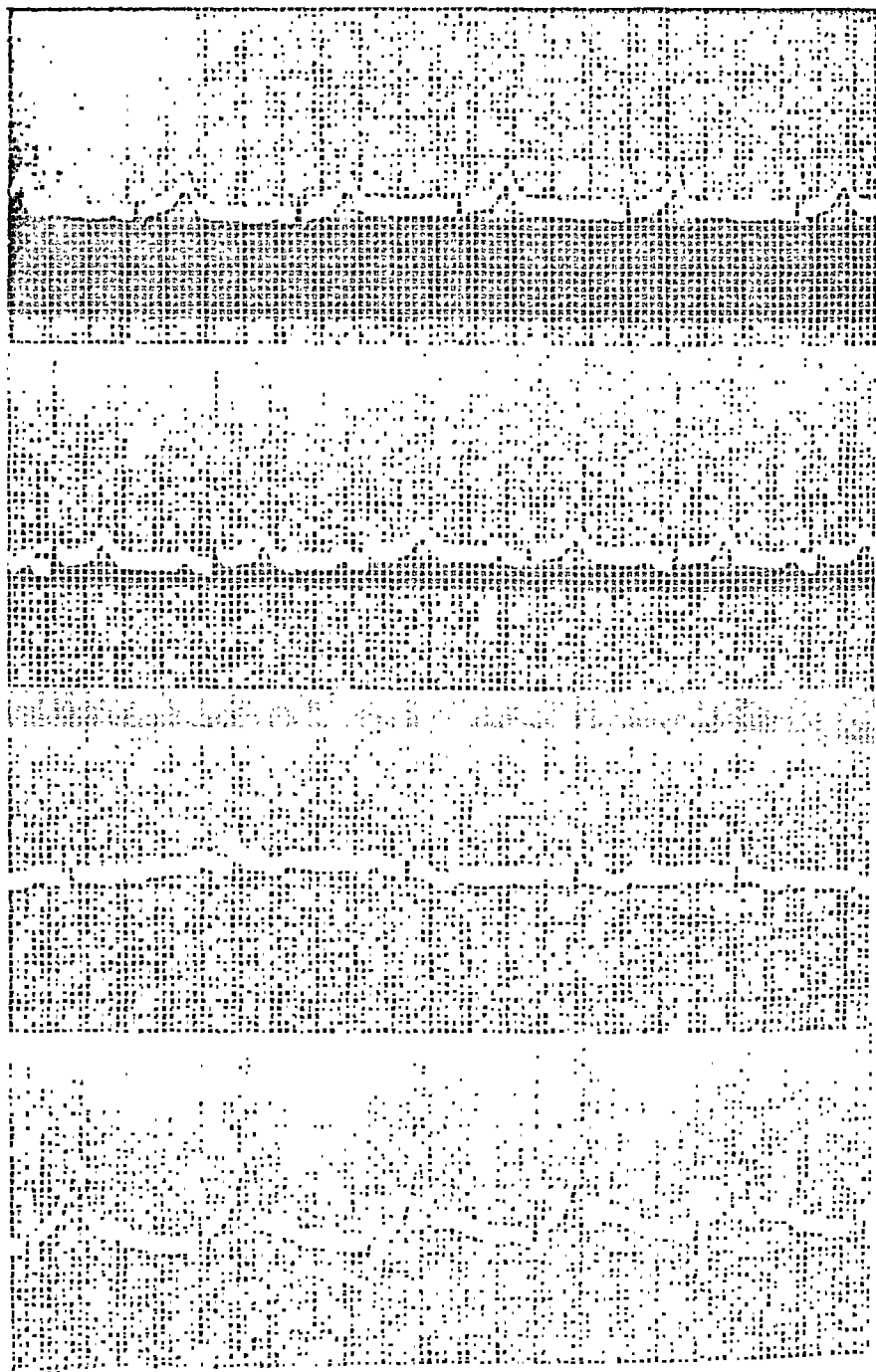


Fig. 5.—Series V taken July 10, 1944.

during the second twenty-four hours in an attempt to maintain the high blood level, but this resulted in a further rise. On the third day with oral medication the level decreased to 14.2 mg. from 21.1 mg. on the previous day. It was maintained at about 6 mg. for the next five days, following which the dosage was reduced again and sulfadiazine therapy discontinued on the twelfth day of hospitalization. There were no apparent ill effects from the drug except for a transient hematuria. Since this cleared in spite of continued sulfadiazine therapy, which was not varied to the slightest degree because of bloody urine, the hematuria may have resulted from hemorrhagic lesions in the kidney due to the meningococcemia itself rather than from a toxic effect of sulfadiazine.

#### COMMENT

One cannot be sure of the cause of the circulatory collapse which occurred in this patient. Recently Hill and Lever<sup>2</sup> have reported three patients who recovered from this shocklike state following treatment with adrenal cortex extract and intravenous glucose-saline solution. However, these authors made no mention of myocarditis or electrocardiographic studies. We were unable to give adrenal cortex extract because it was not available—yet the patient recovered. Myocardial damage alone could explain the sequence of events, but if the circulatory collapse is explained entirely on this basis, the response to adrenalin and large amounts of intravenous fluid is unusual. The Waterhouse-Friderichsen syndrome with hemorrhage into the adrenal glands probably cannot be diagnosed with certainty except by post-mortem examination. However, the course of the illness during the first sixteen hours closely resembled the clinical course of the other cases in which this diagnosis was later proved at autopsy. The only important variation was the recovery of the patient. Pulmonary involvement is occasionally encountered as a complication of meningococcic infection. The physical findings in our patient were those of multiple small areas of consolidation. Whether or not the consolidation was the result of meningococcic bronchopneumonia, widespread embolization or purpura is a question which must go unanswered.

Information is meager regarding the exact nature of the myocardial involvement associated with meningococcemia. The post-mortem records in the literature indicate that myocarditis alone is rare. More commonly there is an associated endocarditis, and occasionally multiple myocardial abscesses have been found. Our patient presented no clinical evidence of endocarditis. The possibility of myocardial abscesses cannot be excluded. However, with extensive purpura in the skin it is more probable that this hemorrhagic process also involved the lungs and myocardium. The series of electrocardiograms and clinical examination show that the patient had and has recovered from severe myocardial damage.

#### SUMMARY

1. A case of recovery from a fulminating meningococcic infection with meningeal, myocardial, and pulmonary involvement has been described.
2. Treatment was symptomatic in addition to large doses of sulfadiazine and large amounts of intravenous fluid to combat the toxemia and shock.

3. Adrenal cortex extract was not administered.

4. Follow-up studies after six months revealed electrocardiographic and clinical recovery.

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## DIASONE: ITS TOXICITY AND THERAPEUTIC EFFECTIVENESS

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**D**IASONE, disodium formaldehyde sulfoxylate diamino-diphenyl-sulfone, was synthesized by one of us (G. W. R.) early in 1937.<sup>1</sup> It was prior to this that our attention was attracted by the reportedly high therapeutic effectiveness of 4,4'-diamino-diphenyl-sulfone\* in streptococcal infection of mice.<sup>2</sup> But the high toxicity of this drug had been an obstacle in its further chemotherapeutic development. For some years past, we had observed the decisive reduction in toxicity obtained by combining arspenamine or other arsenicals with sodium formaldehyde sulfoxylate. There was at the same time only a partial reduction in the therapeutic potency, so that the ratio between the maximum tolerated and minimum therapeutic doses shifted in favor of the new combinations.<sup>3</sup> The resulting product, diasone, proved to be considerably less toxic than the parent substance diamino-diphenyl-sulfone. The effect in streptococcal infection in mice remained appreciable.

It is interesting to note that sulfonamides, and particularly diamino-diphenyl-sulfone, have likewise an effect in experimental tuberculosis. Rich and Follis<sup>4</sup> were the first to find that sulfanilamide has an inhibitory influence on infection in experimental tuberculosis in guinea pigs. It was seen by later investigators that diamino-diphenyl-sulfone has an even better effect in experimental tuberculosis. In 1940, Feldman, Hinshaw, and Moses<sup>5</sup> showed also that a derivative, sodium P,P'-diaminodiphenylsulfone-N,N'-didextrose sulfonate, designated as promin, had similarly an inhibitive effect on experimental tuberculous infection. In January, 1943, Callomon<sup>6</sup> published a paper in which diasone, when compared to promin and other chemical compounds, was shown to produce the most beneficial results in experimental tuberculosis in guinea pigs. Subsequently Feldman, Hinshaw, and Moses<sup>7</sup> found that diasone is less toxic for guinea pigs than diamino-diphenyl-sulfone and that it is of therapeutic value in experimental tuberculosis, though somewhat less effective than diamino-diphenyl-sulfone. Early in 1943 Petter<sup>8</sup> initiated an extensive clinical trial with diasone which indicated that the drug has a therapeutic effect also in the treatment of human tuberculosis. As a result of his treatment of forty-four patients, he observed that all minimal and moderately advanced cases showed improvement and that there was likewise improvement in a considerable number of far-advanced cases.

From the foregoing it may be noted that diasone has its place in the treatment of experimental and possibly clinical tuberculosis. The purpose of this paper is to establish its pharmacologic properties, particularly its toxicity in lower animals, that is, mice, rabbits, and dogs. Its therapeutic effectiveness in

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\*We shall refer to this compound throughout the paper as diamino-diphenyl-sulfone.

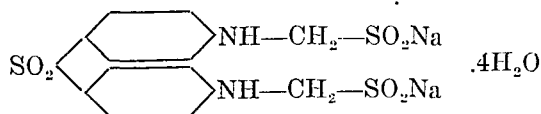
experimental tuberculosis has already been studied by Callomon<sup>6</sup> and also by Feldman, Hinshaw, and Moses.<sup>7</sup> We thought a further extension in the appraisal of its chemotherapeutic properties might be found in the study of the curative effect compared with that of sulfanilamide and sulfadiazine in streptococci and also pneumococci infections in mice.

In beta-hemolytic streptococcus infection, Buttle and co-workers<sup>2</sup> found that the single maximum tolerated oral dose of diamino-diphenyl-sulfone for mice is 5 mg. On the other hand, they reported that the rabbit can be given as much as 2 Gm. per kilogram. The effective therapeutic dose of diamino-diphenyl-sulfone was found by the same authors<sup>2</sup> to be 0.4 mg. In 1938 Bauer and Rosenthal<sup>9</sup> determined that the maximum tolerated oral dose of diamino-diphenyl-sulfone for mice is 0.25 Gm. per kilogram, with the therapeutic activity in streptococci infection of mice thirty times greater than that of sulfanilamide. Feinstone, Bliss, Ott, and Long<sup>10</sup> found diamino-diphenyl-sulfone to be highly toxic for mice and more active than sulfanilamide in streptococcus infection in mice. We have likewise shown that diamino-diphenyl-sulfone was therapeutically active in mice infected with beta-hemolytic streptococcus, but only ten times more so than sulfanilamide.<sup>11</sup>

Diasone is stable and water soluble if protected from oxidation in vacuum glass ampules.<sup>12</sup> Exposed to the air, an aqueous solution after a few minutes turns cloudy. Later a precipitate is formed because of the combined effect of carbon dioxide of the air and the oxidation of diasone, resulting in an increase of the acidity of the solution. By the addition, however, of sodium bicarbonate or sodium hydroxide, the precipitated diasone can be redissolved. If diasone powder is exposed to air for several days, part of it turns insoluble. Mixing diasone and solid sodium bicarbonate (9:1) maintains diasone unchanged and water soluble for a long period of time, provided it is kept in tightly closed bottles.

Gastric juice normally containing from 0.4 to 0.5 per cent hydrochloric acid may act upon diasone, but it was proved by tests that in two hours 0.4 per cent hydrochloric acid at 37° C. causes neither the splitting off of formaldehyde sulfoxylate nor the liberation of diamino-diphenyl-sulfone.

Formed by the reaction between diamino-diphenyl-sulfone and sodium formaldehyde sulfoxylate, diasone cannot be recrystallized and therefore contains some amount of admixture in addition to the major product. The major product represents from 92 to 93 per cent of the substance and corresponds to the following formula:



The balance comprises uncombined sodium formaldehyde sulfoxylate and sodium sulfate.<sup>12</sup> Diasone is sufficiently pure and uniform to be satisfactory in treating infected animals and human beings as well. This drug can be quantitatively estimated by diazotization and subsequent formation of a dye, according to a modification of a method of Bratton and Marshall.<sup>13</sup>

## BIOLOGIC DATA ON DIASONE

Dissolved in distilled water, diasone was orally administered to fifty-two mice. As may be seen from Table I, 100 per cent of the animals which received 2 and even 3 Gm. of the drug per kilogram body weight survived. When 4 Gm. per kilogram were administered to each of fifty-six animals, 82 per cent survived. Of a group of thirty-six mice given 5 Gm. per kilogram 53 per cent survived. Several mice received 6 Gm. per kilogram, a dose which proved lethal. The conclusion may be drawn that for mice the toxicity of diasone is very low.

TABLE I  
TOXICITY OF DIASONE IN AQUEOUS SOLUTION ADMINISTERED ORALLY TO MICE\*

DOSE PER KILOGRAM (GM.)	NUMBER OF ANIMALS	SURVIVALS	
		NUMBER	PER CENT
2.0	18	18	100
3.0	34	34	100
4.0	56	46	82
5.0	36	19	53
6.0	5	0	0

\*Each animal received a single dose.

The chronic toxicity of diasone was investigated on fifty mice to which the drug in food was administered for twenty-one consecutive days. Of these animals, 90 per cent survived a diet containing  $\frac{3}{4}$  per cent of diasone (Table II). A diet containing  $\frac{3}{4}$  per cent of sulfadiazine was tolerated by only 60 per cent, while a 1 per cent diet was lethal to all of ten animals.

TABLE II  
CHRONIC TOXICITY OF DIASONE FED TO MICE FOR TWENTY-ONE DAYS

CONCENTRATION OF DRUG IN FOOD (PER CENT)	NUMBER OF ANIMALS	SURVIVING 7 DAYS		SURVIVING 14 DAYS		SURVIVING 21 DAYS	
		NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
$\frac{1}{4}$	10	10	100	9	90	9	90
$\frac{1}{2}$	20	18	90	17	85	17	85
$\frac{3}{4}$	10	10	100	10	100	9	90
1.0	10	8	80	7	70	6	60

Studies on twenty-five rats indicated that more than 7 Gm. of diasone per kilogram could be tolerated, a further manifestation of the drug's low toxicity (Table III).

TABLE III  
TOXICITY OF DIASONE IN AQUEOUS SOLUTION ADMINISTERED ORALLY TO RATS

DOSE PER KILOGRAM (GM.)	NUMBER OF ANIMALS	SURVIVALS	
		NUMBER	PER CENT
3.0	5	5	100
4.0	5	5	100
5.0	5	5	100
6.0	5	5	100
7.0	5	5	100

Rabbits do not generally tolerate chemical compounds as well as do mice or rats. Experiments were performed on twenty-nine rabbits with the result that 100 per cent survived 2.5 Gm. per kilogram, while 83 per cent tolerated 3 Gm.

(Table IV). Eighty per cent of the animals withstood 3.5 Gm., but only 40 per cent could tolerate 4 Gm. of the drug orally. It appears that the maximum tolerated dose is therefore between 3 and 3.5 Gm. per kilogram.

TABLE IV  
TOXICITY OF DIASONE ADMINISTERED ORALLY TO RABBITS

DOSE PER KILOGRAM (GM.)	NUMBER OF ANIMALS	SURVIVALS	
		NUMBER	PER CENT
1.0*	3	3	100
1.5	2	2	100
2.0	3	3	100
2.5	5	5	100
3.0	6	5	83
3.5	5	4	80
4.0	5	2	40

\*Each dose was dissolved in 100 c.c. of water.

We were interested to note that rabbits also tolerated well an intravenous administration of diasone. Each of eighteen rabbits receiving 1 Gm. per kilogram intravenously survived without loss of weight for an average period of thirty days (Table V). An increase of the dose to 1.5 Gm. gave a 40 per cent survival and 2 Gm. a 33 per cent toleration. By contrast, sodium sulfadiazine could be given in only a  $\frac{1}{2}$  Gm. dose per kilogram. Larger doses were not tolerated.

TABLE V  
TOXICITY OF DIASONE ADMINISTERED INTRAVENOUSLY TO RABBITS

DOSE PER KILOGRAM (GM.)	NUMBER OF ANIMALS	SURVIVALS	
		NUMBER	PER CENT
1.0	8	8	100
1.5	5	2	40
2.0	3	1	33

Drug given in a 10 per cent solution, containing 10 per cent sodium bicarbonate. The last-named substance was added for the purpose of holding the drug in solution. Otherwise in the presence of air the solution becomes oxidized and cloudy.

In conclusion, it may be stated that the chronic and acute toxicity of diasone tested orally and intravenously on mice, rats, and rabbits is far less than that of most well-known sulfonamides, including sulfadiazine.\*

From Table VI it is readily seen that dogs tolerate diasone in large daily oral doses for long periods of time. By way of comparison, it should be noted that the dose found practical at present and generally used by clinicians investigating the treatment of tuberculosis in human beings is about 0.015 Gm. per kilogram (approximately 1 Gm. of diasone per day). For sixty consecutive

\*F. T. Callomon was kind enough to make histopathologic studies which may shed some light on deaths occurring among mice when fed diasone. The following is quoted: "At  $\frac{1}{4}$  per cent drug diet there were no changes from the normal except for some focal epithelial alteration in a few convoluted tubules with some hyaline casts in the tubules. At  $\frac{1}{2}$  per cent drug diet obvious epithelial damage was found in some dilated tubules, such as swelling and vacuolization of the cells which showed indistinct outlines, pale nuclei, and were partly anuclear. Hyaline casts and some leucocytic infiltration were observed. At  $\frac{3}{4}$  per cent drug diet these changes became more obvious than at  $\frac{1}{2}$  per cent drug diet, for a greater part of the convoluted tubules, some of Henle's tubules, and also some collecting tubules were afflicted. Cellular and hyaline casts were found in many tubular spaces, while the capsular spaces were not obviously dilated, containing sometimes some hyaline precipitate. In the cortical district leucocytic infiltration was observed around or between some damaged, partly collapsed tubules. Spleens and livers of the mice were normal at  $\frac{1}{4}$  per cent and  $\frac{1}{2}$  per cent drug diet, while cloudy swelling was observed in the livers, and a moderate reduction of the lymphocytes of the mantle zone with hyperplasia of germinal centers in the spleens of the mice . . . neither in the mouse nor dog kidneys were gross concretions (uroliths) found and no corresponding destruction of the renal tissue."

TABLE VI  
CHRONIC TOXICITY OF DIASONE ADMINISTERED ORALLY TO DOGS

DOG	DAILY DOSE (GM. PER KILOGRAM)	TOTAL DAILY DOSE	NUMBER OF ADMINISTRATIONS
1	0.050	0.850	60
2	0.050	0.725	60
3*	0.035	0.500	180
4	0.035	0.500	190
5	0.300	3.5	110
6†	0.300	3.5	100

No diarrhea or vomiting was observed in any of the animals.

\*Gave birth to four puppies and raised them to mature state.

†Gave birth to eight puppies and raised them to mature state.

days, Dogs 1 and 2 received a daily dose of 0.05 Gm. per kilogram, which is three times larger than the daily human dose. These animals were sacrificed, and at autopsy the kidneys were seen to be only slightly affected. Dogs 3 and 4 received for 170 days a dose of 0.035 Gm. per kilogram per day, more than twice the therapeutic human dose. The animals did not lose weight and showed no signs of toxic effects. They were obviously in vigorous health and maintained a normal appetite. Dog 5 was given daily a large dose of 0.3 Gm. per kilogram, equivalent to twenty therapeutic human doses. The dog's original weight at the inception of the experiment was 22 pounds, but after ninety-five daily administrations of diasone it increased to 26 pounds. Concentrations of the drug in blood were estimated periodically four hours after the administration of the drug and were found to average 2 mg. of diasone per 100 c.c. of blood. Considerable quantities of diasone were found in the urine and lesser amounts in the feces. Dog 6 also received a daily dose of 0.3 Gm. per kilogram for eighty-three days. No diarrhea, vomiting, or other untoward symptoms were observed. The urine was negative for albumin and sugar, the weight was well maintained, and the health of the animal appeared good. It is interesting to notice that the prolonged administration of diasone did not unfavorably influence the course of pregnancy in Dogs 3 and 6. The first of these had a healthy litter of four, the second a litter of eight. The young were suckled by their mothers and grew into vigorous animals.

#### BLOOD LEVELS AND URINARY EXCRETION OF DIASONE

Blood levels were determined in mice that received food containing  $\frac{1}{2}$  per cent diasone for anywhere from four hours to six consecutive days. Since each of the animals had to be sacrificed in order to supply the necessary quantity of blood, different mice were used at various time intervals. The total blood levels ranged only from 2.0 to 3.8 mg. per 100 c.c. of blood (Table VII).

Mice that received orally only one dose of 0.01 Gm. of diasone (approximately 0.5 Gm. per kilogram) maintained fairly uniform blood levels during twenty-four hours, after they reached the highest point in the first six hours (Table VIII).

A rabbit whose oral intake of diasone was one dose of 1 Gm. per kilogram, or a total of 2.75 Gm., also showed a low but consistently uniform blood level during twenty-four hours. A low blood level, however, does not indicate an in-



TABLE VII

DETERMINATION OF BLOOD LEVELS IN MICE FED A DIET CONTAINING  $\frac{1}{2}$  PER CENT DIASONE

BLOOD EXAMINED*	MG. PER 100 C.C. BLOOD		
	FREE	CONJUGATED†	TOTAL
4 hr. after starting drug	1.31	Not tested	
4 hr. after starting drug	0.6	Not tested	
24 hr. after starting drug	1.32	0.68	2.0
24 hr. after starting drug	1.46	0.84	2.3
48 hr. after starting drug	3.1	0.7	3.8
48 hr. after starting drug	2.0	1.1	3.1
6 days after starting drug	1.63	1.27	2.9
6 days after starting drug	1.63	1.27	2.9

\*Each experiment was performed on a different animal, sacrificed for the necessary amount of blood.

†Conjugated refers to results obtained upon boiling the blood filtrate with 20 per cent para-toluene sulfonic acid.

TABLE VIII

## BLOOD LEVELS

DIASONE IN AQUEOUS SOLUTION ADMINISTERED ORALLY TO MICE  
ONE DOSE OF 0.01 GM. PER KILOGRAM

BLOOD EXAMINED*	MG. PER 100 C.C. BLOOD	
	(TOTAL)	
2 hr. later	2.18†	
2 hr. later	3.43†	
6 hr. later	2.76	
6 hr. later	2.19	
18 hr. later	1.00	
18 hr. later	2.18	
24 hr. later	2.00	
24 hr. later	1.64	

\*Each experiment was performed on a different animal, sacrificed for the necessary amount of blood.

†Conjugated not included.

complete absorption, since the urinary excretion of diasone reached 603 mg. in the twenty-four hour specimen and 324 mg. in the second-day specimen (Table IX). During the following nineteen days, the drug continued to be eliminated in the urine. After nineteen days, the total reached 1.194 Gm. Since we cannot account for a total of 2.75 Gm. of diasone ingested, it must be assumed that the drug was partially destroyed in the body or was changed into a chemical compound which could not be determined by the colorimetric method used. Comparatively small amounts were found in the feces.

Because of the large dosage, the highest blood levels were obtained in rabbits which received orally 0.5 Gm. per kilogram daily for a period of twenty days (Table X). The blood specimens were taken at two, four, and twenty or twenty-four hours after drug administration. The total blood levels (with one exception) ranged from 6.4 to 16 mg. per 100 c.c. shortly after drug intake, while at the end of the day the blood levels varied from 3.5 to 5.2 mg. In order to obtain higher blood levels, several rabbits received orally single doses of from 3.5 to 4 Gm. of diasone. As a result, the total blood levels ranged from 4.8 to 11.7 mg. per 100 c.c.\*

Intravenous injection into a rabbit of a single dose of 0.2 Gm. per kilogram produced a moderately high blood level. After the first twenty-four hours

\*The conjugated fraction of blood levels, following intravenous injections of diasone into rabbits, appears to be irregular, with a tendency to be higher in the first four hours.

TABLE IX  
BLOOD LEVELS AND ELIMINATION IN URINE

DIASONE ADMINISTERED ORALLY TO A RABBIT—ONE SINGLE DOSE OF 1 GM. PER KILOGRAM\*

TIME AFTER ADMINISTRATION	BLOOD (MG. IN 100 C.C.)			URINE (MG. IN 24-HOUR SPECIMEN)		
	FREE	CONJUGATED†	TOTAL	FREE	CONJUGATED†	TOTAL
2 hr.	1.15	0.65	1.80			
4 hr.	1.38	0.64	2.02			
24 hr.	1.52	1.15	2.67	439.37	163.83	603.40
48 hr.				256.28	67.60	324.48
3 days				62.56	21.62	84.18
4 days				29.58	18.91	48.49
5 days				25.20	6.80	32.00
6 days				11.18	11.52	22.70
7 days				7.6	2.15	9.75
8 days				2.06	2.84	4.90
9 days				2.97	1.08	4.05
10 days)						
11 days)				9.75	4.16	13.91
12 days)						
13 days)						
14 days)				27.47	18.87	46.34
15 days)						
16 days)						
17 days)						
18 days)				Trace		
19 days)						
Total				874.52	319.38	1194.20

\*A total of 2.75 Gm. of diasone.

†Conjugated refers to results obtained upon boiling the blood filtrate with 20 per cent para-toluene sulfonic acid.

TABLE X  
DETERMINATION OF BLOOD LEVELS IN RABBITS

DIASONE GIVEN ORALLY 0.5 GM. PER KILOGRAM DAILY FOR A PERIOD OF TWENTY DAYS

WEIGHT (GM.)	DATE	BLOOD TAKEN	BLOOD LEVELS (MG. PER 100 C.C.)		
			FREE	CONJUGATED*	TOTAL
First rabbit					
1,900	6/30/43	2 hr.	4.0	10.40	14.40
		6 hr.	5.25	3.40	8.65
		20 hr.	1.60	2.40	4.0
		After 15th dose			
1,580	7/ 8/43	2 hr.	1.52	5.28	6.80
		4 hr.	3.36	11.64	15.00
		24 hr.	3.40	1.08	4.48
		After 20th dose			
Second rabbit					
1,940	6/30/43	2 hr.	1.55	12.45	14.00
		4 hr.	1.90	14.10	16.00
		20 hr.	1.60	1.90	3.5
		After 15th dose			
1,840	7/ 8/43	2 hr.	1.6	1.40	3.0
		4 hr.	1.28	5.14	6.42
		24 hr.	1.02	4.18	5.20
		After 20th dose			

\*Conjugated refers to results obtained upon boiling the blood filtrate with 20 per cent para-toluene sulfonic acid.

the urine of the same rabbit contained substantial quantities of diasone. During the six days following, only small quantities were excreted, with a final total of 272.4 mg. A very small amount was found in the feces. The amount excreted again did not equal the intake.

On the other hand, high blood levels, with no conjugated fraction present, were obtained when a larger dose of diasone amounting to 1 Gm. per kilogram was administered intravenously to a rabbit. These blood levels ran from 18.4 to 23.6 mg. per 100 c.c. The urinary excretion continued for eight days; it totaled 527.9 mg. of diasone, or 26 per cent of the amount administered.

When diasone was given orally to dogs in daily quantities of 0.035 Gm. per kilogram, the blood levels were found to be fairly low (from 0.7 to 1.2 mg.), and no conjugated fraction was present. When a larger daily dose of 0.3 Gm. per kilogram was administered, the blood levels reached 3.2 mg. and in one instance 7.8 mg.

The urinary excretion of diasone in dogs which received 0.035 Gm. per kilogram was found to be between 23.9 and 81.4 mg. in a twenty-four hour specimen (Table XI). On the other hand, the two other dogs whose intake of the product was considerably higher showed in their twenty-four hour urinary excretion from 107.3 to 544 mg. of the drug. It must be noted again that, as in the case of the rabbits, the quantities of the drug ingested were not commensurate with its elimination.

TABLE XI  
URINARY EXCRETION OF DIASONE BY DOGS

DOSE	TIME AFTER ADMINISTRATION (HR.)	NUMBER OF DAYS AFTER START OF EXPERIMENT	URINE	FECES
			MG. IN 24-HR. SPECIMEN (FREE)	MG. IN 24-HOUR SPECIMEN (FREE)
0.035	24	103	81.42	55.82
0.035	24	106	39.13	30.60
	24	160	23.98	32.18
0.3	24	63	107.30	201.96
	24	112	544.00	75.95
0.3	24	64	117.9	143.6

Urine contained no albumin or sugar.

#### THERAPEUTIC EFFECTS OF DIASONE

In all of the therapeutic experiments, drugs were administered to mice in food according to the method described by Litchfield, White, and Marshall.<sup>14</sup> Mice were infected with the beta-hemolytic streptococcus strain C203, using 1,000 minimum lethal doses. By a lethal dose is meant one containing sufficient bacteria of a certain virulence which would eventually kill mice in the course of several days. It was necessary, therefore, to infect mice with 1,000 minimum lethal doses in order to kill the animals in the first twenty-four to forty-eight hours. From Table XII one can see, for example, that the ten control mice died on the first day. Those fed the  $\frac{1}{4}$  per cent diasone diet showed a therapeutic effect superior to that exerted by a diet containing  $\frac{1}{4}$  per cent sulfanilamide. But with a diet of  $\frac{1}{2}$  per cent sulfanilamide, the level at which the optimum effect of the sulfonamide is produced, animals show a better survival rate than with diasone.

TABLE XII

COMPARATIVE THERAPEUTIC EFFECTS OF DIASONE AND SULFANILAMIDE ON MICE INFECTED WITH BETA STREPTOCOCCUS HEMOLYTICUS STRAIN C203

PER CENT OF DRUG IN FOOD	DRUG	NUMBER OF MICE USED	PER CENT OF SURVIVALS									
			DAYS									
			1	2	3	4	5	6	7	14	21	28
1/4	Diasone	20	95	85	75	75	75	75	65	65	65	
1/2		15	87	87	80	73	67	60	60	47	47	47
1/4	Sulfanilamide	10	90	40	40	40	40	40	40	40	30	30
1/2		10	100	100	70	70	70	70	70	70	70	70
	Controls	10	10	0								

From Table VII one can see that in mice receiving 1/2 per cent diasone the average blood level is indicated as 2.83 mg. per 100 c.c. of blood.

There is no information available in literature concerning blood levels in animals receiving 1/4 or 1/2 per cent sulfanilamide drug diet. It was found that blood concentration in mice given a 1 per cent sulfanilamide drug diet is 11.6 mg. per 100 c.c. (Feinstone and others, Bull. Johns Hopkins Hosp. 67: 131, 1940.)

In our experiments on type II pneumococcus infection, we used strain M-48, as described in a previous publication.<sup>15</sup> Mice were infected intraperitoneally with ten minimum lethal doses containing about 250 bacteria. From Table XIII it may be noted that a diet containing 1/4 per cent diasone had a considerably greater therapeutic effect than did a diet of 1/4 per cent sulfadiazine. With a 1/2 per cent drug diet of sulfadiazine, however, the survivals were somewhat higher than in the case of diasone.

TABLE XIII

COMPARATIVE THERAPEUTIC EFFECTS OF DIAZONE AND SULFADIAZINE ON MICE INFECTED WITH PNEUMOCOCCUS TYPE II STRAIN M-48

PER CENT DRUG IN FOOD	DRUG	NUMBER OF MICE USED	PER CENT OF SURVIVALS									
			DAYS									
			1	2	3	4	5	6	7	14	21	28
1/4	Diasone	20	100	95	95	90	90	75	55	40	40	40
1/2		30	100	93	93	93	87	83	80	50	43	43
1/4	Sulfadiazine	40	95	75	50	33	25	25	15	5	5	5
1/2		40	100	100	100	100	93	93	88	58	55	55
	Controls	15	27	0								

See footnote to Table XII for diasone blood level.

The average blood level of sulfadiazine administered to mice receiving 1/2 per cent drug in the diet was found to be 30.6 mg. per 100 c.c. (Feinstone and others: Bull. Johns Hopkins Hosp. 67: 435, 1940.)

## SUMMARY

In general conclusion it may be stated that the chronic and acute toxicity of diasone tested orally and intravenously on mice, rats, and rabbits is noticeably less than that of most well-known sulfonamides, including sulfadiazine. It is far less toxic than diamino-diphenyl-sulfone. Diasone has shown high therapeutic effectiveness in mice infected with beta-hemolytic streptococcus as well as type II pneumococcus infection. At the present time, diasone is being investigated clinically in the treatment of tuberculosis.

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ment. Blood films were made from the webb on the seventh experimental day in the first experiment and in both experiments at the time of death of each duck. These were stained with Wright's modification of Leishman's Romanowsky stain. Counts were made on five or more oil immersion fields in different parts of each film until a minimum of 500 cells had been counted. Cells classed as immature were chosen on the basis of three criteria: nuclear structure, nuclear shape, and the extent of cytoplasmic chromation.

The ducks were sacrificed by clamping the necks. An autopsy was performed immediately thereafter and the tissues were placed in both Bouin's solution and a 10.0 per cent solution of formalin. Those fixed in the former preservative were used for histopathologic studies. The sections were stained with hematoxylin and eosin. Bone marrow for sectioning was obtained from the femur.

Imprint (dab) films were made at the time of autopsy from the liver, spleen, rib, and the femur. These were stained with Wright's modification of Leishman's Romanowsky stain. They were examined under oil immersion, care being given in those of the liver and spleen, particularly, to note evidence of erythropoiesis. Counts were made of the phagocytic cells present and an estimate was made of the amount of phagocytosed material they contained in each case. Films of bone marrow were studied carefully for evidence of changes in plasticity in the erythrocytic series.

The number of ducks used and the time that each experimental procedure was carried out is given in the different experiments.

#### EXPERIMENTS

*Experiment 1.*—Six ducks, 3 weeks of age, were used. Four were given cobalt and two were kept as the controls. The total number of red blood cells and the time of the injection of the cobalt are given in Fig. 1. These birds were killed on the fourteenth day following the first injection of cobalt. The erythrocyte numbers increased by 22 to 38 per cent in the ducks given cobalt (Fig. 1). No significant variation occurred in the number of red blood cells in the two control birds during this same interval.

*Experiment 2.*—Fifteen ducks, 3 weeks of age, were used. Ten were given 4 mg. of cobalt daily, while five birds were kept as controls. On the third day of the experiment, three of the ducks given cobalt and one untreated bird were killed; on the seventh experimental day three ducks given cobalt and one control were killed; on the nineteenth experimental day two ducks given cobalt and one control were killed; on the twenty-eighth day of the experiment the remaining two cobalt-treated ducks and two controls were killed.

Fig. 2 gives the average number of red cells for the second group of ducks at each time they were counted. The red blood cells increased in the ducks given cobalt, as shown in Fig. 2, until about the eleventh day; from this time until the twenty-eighth day the total number of red cells remained approximately constant. The number of cells during this interval was increased by 38.0 per cent over the number present at the beginning of the experiment. The average number of erythrocytes for the control birds remained practically constant for the first eleven days of the experiment. At this time the number of

red cells increased, reaching their maximum number on the twenty-first day of the experiment, when there was an increase of 15 per cent in the number of red cells of the control animals as compared with the number at the beginning of the experiment.

The average weight of the birds in the control and the experimental groups is given in Fig. 2. There was a progressive increase in the weight of all birds. The average increase of those in each group was approximately the same.

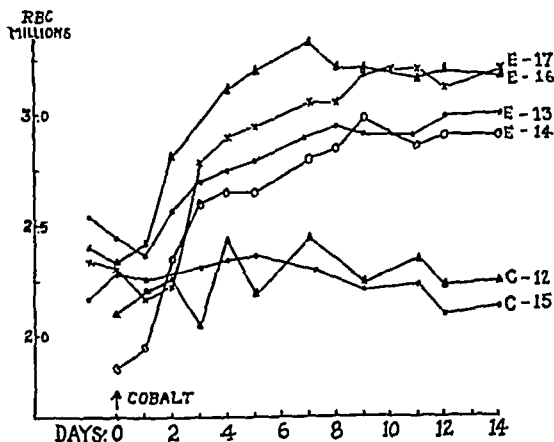


Fig. 1.—The effect of daily injections of cobaltous chloride on the red blood cell counts of young ducks. E, Experimental cobalt-treated ducks C, Control ducks, untreated.

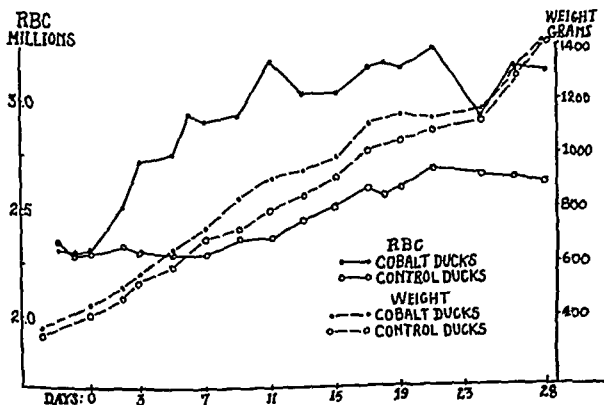


Fig. 2.—Average erythrocyte numbers and body weights of a cobalt-treated group and a control group of ducks. At the start of the experiment the cobalt-treated group consisted of ten ducks and the control group of five ducks.

## HEMATOLOGIC STUDIES

*Blood Films.*—In Table I are given the number of cells counted and the percentages of immature cells found, arranged in terms of experimental days. With the exception of Duck 13, the general trend is toward an initial rise in the percentages of young red cells during the first three days of cobalt injection, followed by a continuous decline in these percentages with continued medication. The control animals showed some decline but much less than did the experimental ducks. With the fall in young cell percentages in the experimental animals, there was increasing nuclear degeneration and pyknosis, cellular fragmentation, and the appearance of cells with highly immature nuclei and cytoplasm showing primitive basophilia. These latter changes were not found to accompany the slight decrease in young cell percentage in the control birds.

*Films From Liver and Spleen.*—Films made from the liver and spleen gave data which were supplementary with one exception. They showed the extent of phagocytic activity, and in one case there were signs of extramedullary erythropoiesis in the liver.

These preparations in both groups showed more normal, mature red cells in the experimental than in the control animals. While this is a subjective estimate, it may indicate that these organs in the experimental animals were more congested with blood.

TABLE I  
PERCENTAGES OF YOUNG ERYTHROCYTES IN SMEARS OF PERIPHERAL BLOOD

EXPERI- MENTAL DAYS	DUCK	GIVEN COBALT CHLORIDE	TOTAL NUMBER OF CELLS COUNTED	PERCENT- AGE OF IMMATURE CELLS	REMARKS
3	111	+	884	7.2	Some primitive nuclei
	114	+	692	6.8	Some primitive nuclei
	119	+	732	8.0	A few atypical cells
	123	0	902	6.2	
7	12	0	1,210	6.6	
	13	+	659	5.8	Pyknosis, degeneration
	14	+	668	5.7	
	15	0	852	6.7	
	16	+	686	4.0	Much degeneration
	17	+	820	5.6	Some degeneration
	115	+	788	3.1	Much cellular debris
	121	0	677	6.0	
	117	+	574	2.9	Many primitive nuclei
	116	+	719	3.4	A few polychromatophils
14	12	0	920	8.1	
	13	+	963	28.4	Extreme fragmentation; many very primitive cells, some showing pyknosis
	14	+	505	2.0	Pyknosis
	15	0	620	9.9	
	16	+	681	3.5	Degeneration
	17	+	780	2.8	Nuclear degeneration; no polychromatophils
	112	+	729	2.2	Much fragmentation
	122	0	602	5.8	
19	113	+	612	2.8	Very few polychromatophils
28	109	+	1,024	1.6	Very much cellular debris
	110	+	824	3.0	Many atypical cells
	120	0	712	5.1	Young cells primitive
	124	0	698	5.7	

Phagocytic cells in both hepatic and splenic smears from birds killed on the third and seventh days were slightly more numerous in the experimental animals than in the controls. A striking observation, however, was the much smaller amount of phagocytosed material in the cells of the experimental birds which were killed on the fourteenth, nineteenth, or twenty-eighth days. The difference was too great to be accounted for by the slight increase in number of cells and is particularly striking in the light of the much greater amount of debris found in peripheral blood of these experimental birds.

In Duck 13, killed on the fourteenth day of the first experiment, there were found, in the imprint smear of the liver, numerous erythroblasts which were primitive enough to show no polychromatophilia. Since these cells were not found in the peripheral circulation, it is assumed that their presence in the liver indicates some hepatic erythropoiesis in this bird. It will be recalled that this bird is the one that, at time of autopsy, showed an extremely elevated percentage of immature cells (Table I).

*Marrow Preparations.*—In all birds, films were made from both rib and leg marrow. Findings in every case differed slightly in the rib and leg marrow from the same bird. In the controls, there was evidence of slightly more active erythropoiesis in the rib marrow. In the experimental animals the rib marrow showed changes more pronouncedly than did the leg marrow, but, after the fourteenth day, these changes were progressively hypoplastic.

In the first group of birds which was killed on the fourteenth day, the rib and leg marrow of the experimental animals showed some stimulation of red cell production with only one essential difference; that of the leg showed a mild, typical hyperplasia; that of the rib showed an increase in more mature cells of the erythropoietic series but was impoverished in the basophilic and early polychromatophilic erythroblasts. This difference was noted also in the leg marrow of Duck 13, where the hyperplasia of the late erythropoietic elements was very great, but the early stages were entirely absent.

In the second experiment the changes described occurred progressively in the experimental animals killed on the nineteenth and twenty-eighth days. In each bird the changes noted were more advanced in the rib as compared to the leg marrow. The rib marrow of Ducks 109 and 110, killed on the twenty-eighth day, showed a generalized hypoplasia of the erythropoietic series which seemed particularly advanced in Duck 109. The leg marrow reflected the same change clearly, but in both birds there was an indication of plasticity confined largely to the acidophilic erythroblasts.

#### PATHOLOGIC STUDIES

Each of the ducks appeared normal at the time it was sacrificed. The skin over the back was desquamated at the site of the injections and the underlying subcutaneous tissue frequently was indurated. The pathologic lesions here were those of an acute and chronic reaction.

No gross changes were observed in the organs of any of the ducks. The weight of the organs of the birds in the experimental group did not vary significantly from the controls. The femur bone marrow from the ducks given cobalt for three-, seven-, and fourteen-day periods was more red in color and



semiliquid when compared to that of the corresponding controls. The marrow sections from the ducks injected with cobalt for twenty-eight days did not show microscopically a significant difference from that of the controls (Fig. 3). In the hyperplastic bone marrow, the predominating cell was large and mononuclear; the nucleus was usually either round or oval. The nucleus stained lightly with

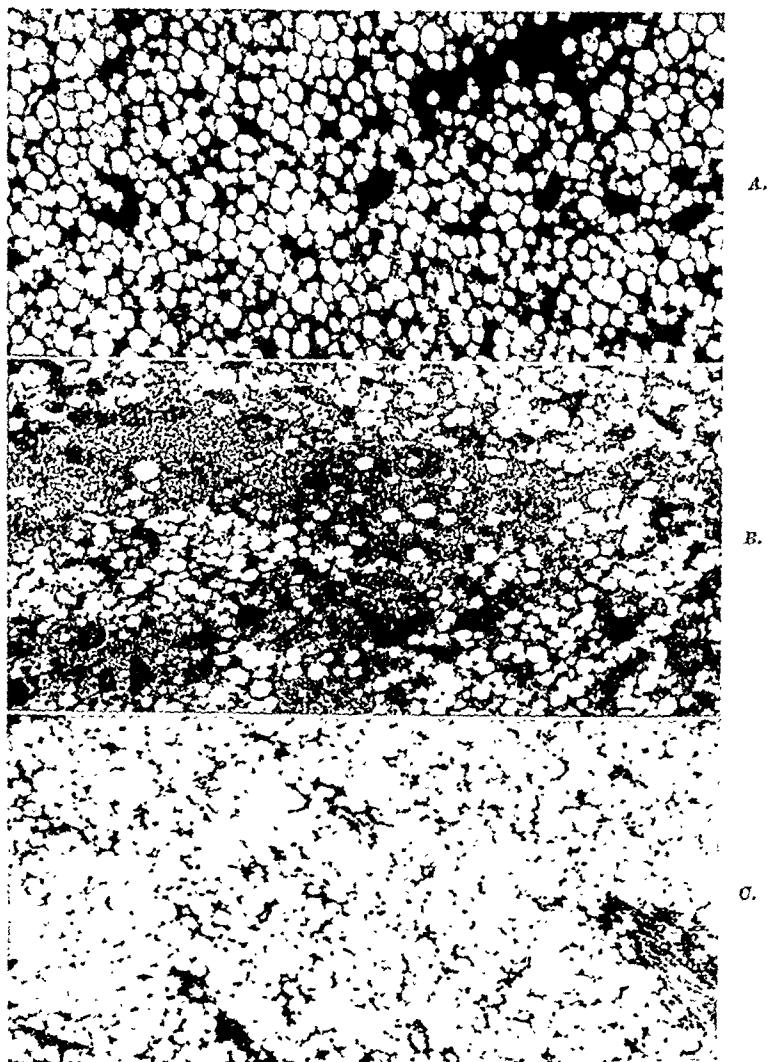


Fig. 3.—A, Femur marrow from Duck 121. This is a normal duck and is the control for Duck 116 shown in B. B, Femur marrow from Duck 116 given cobalt for seven days. The marrow is hyperplastic. C, Femur marrow from Duck 110 given cobalt for twenty-eight days. The marrow is hypoplastic.

hematoxylin in some of the sections, while in others it stained deeply. The cytoplasm varied in its staining reaction from a light pink to a deep red. In some of the birds the cytoplasm of these marrow cells was filled with large red particles.

There was an increase in the number of cells in the masses of extramedullary hemopoietic tissue in the sections of lungs, liver, adrenals, and kidney from the

ducks given cobalt for the three-, seven-, and fourteen-day periods. The control ducks of corresponding age showed few, if any, extramedullary foci of blood-forming tissue in the liver and kidney. The experimental ducks had large foci of these cells protruding into the lumina of the blood sinuses (Figs. 4 and 5). These cells were mononuclear and dark staining. In the lumina of some of the large blood vessels, groups of cells were present similar to those forming the masses projecting into the lumen. It was obvious from microscopic study that

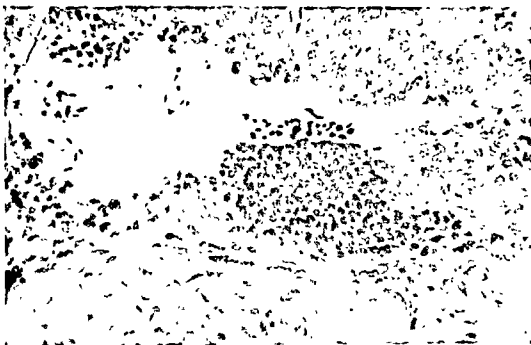


Fig. 4.—Hyperplasia occurs in the extramedullary erythroblastic tissue in the ducks given cobalt for a short period. The adrenal from Duck 111 given cobalt for three days. Note the projection of these cells into the lumen of the blood vessel.



Fig. 5.—Hyperplasia occurs in the extramedullary erythroblastic tissue in the kidney similar to that in the adrenal. Duck 114 was given cobalt for three days.

these were neither agglutinated red cells nor groups of normal circulating white blood cells.

There was an apparent increase in the number of cells in the splenic pulp of ducks given cobalt. The increase occurred in the number of large mononuclear cells, many of which had eosinophilic particles in their cytoplasm. Hyperplastic

masses of these reticular-like cells projected into the lumen of the splenic sinuses in a manner similar to those in the liver, kidney, and adrenal.

#### DISCUSSION

Polycythemia may be produced in the duck following the subcutaneous injection of cobaltous chloride. The increase in the number of cells in the erythroblastic series is similar to that occurring in mammals. As far as we know, this is the first time results have been published in which birds have been used for the study of polycythemia produced by cobalt.

In this experiment the increase in the total number of red cells rapidly progressed until the fourteenth day. From this time until the end of the experiment on the twenty-eighth day the number of red cells remained approximately constant. The number of cells during this interval remained at about 38 per cent over that at the beginning of the experiment. It is of interest to observe that the red cells of the controls increased about 16 per cent during the same period. These observations suggest that the normal erythrocyte count for young ducks gradually increases with age from 3 to 6 weeks. The average number of red cells for 3-week-old ducks is 2.3 million. Hewitt observed a variation in the total number of red blood cells in young and adult ducks.<sup>10</sup>

The percentage of young erythrocytes is increased in the peripheral blood during the first few days following the initial injections of cobalt. A number of these young cells were found in a dab smear of the liver of Duck 13 on the fourteenth experimental day. The histologic studies of the tissues corroborate the hematologic observations of hyperactivity of the erythroblastic series. The bone marrow from the femur was hyperplastic in the ducks killed on or before the fourteenth day of the experiment. The extramedullary foci in the spleen, liver, adrenal, and kidney also showed a greater number of cells during this same period as compared with the control birds. It appears, therefore, that all the erythroblastic foci are stimulated within three days following the commencement of the subcutaneous injections of cobalt.

The ducks which were given cobalt showed a sustained polycythemia from the fourteenth day to the end of the experiment on the twenty-eighth day. During this time there was a gradual diminution in the number of young red blood cells in the peripheral blood. The femur marrow from the ducks given cobalt and sacrificed on either the nineteenth or twenty-eighth day of the experiment appeared the same in histologic sections as those of the controls of similar age. It was definitely hypoplastic as compared with the femur marrow from the birds given cobalt and sacrificed on the third, seventh, or fourteenth day. Furthermore, the foci of extramedullary erythropoiesis were absent in the ducks given cobalt and sacrificed on the nineteenth and the twenty-eighth days of the experiment. The hematologic and pathologic observations indicate that cobalt at first stimulates erythropoiesis, after which it may act either as a depressor or an injurious agent to the erythroblastic series.

Some consideration must be given the fact that total red cell counts persisted while the percentages of young cells were falling and the marrow showed an increasing hypoplasia. The mechanism of cobalt polycythemia has been studied by several workers. According to Orten, the general opinion seems to

be that in rats, rabbits and dogs, this polycythemia is a product of increased blood production rather than of decreased blood destruction.<sup>7</sup> In our animals, this stimulation of cell production would seem to be a feature of the early stages of cobalt administration. With the continuation of cobalt, however, there is a fall in erythropoietic activity not reflected in the total red cell counts of the peripheral blood. At the same time that the production of young cells is depressed, there is a depression in the phagocytic activity, or capacity, of the reticulo-endothelial elements of the spleen, particularly, and of the liver. At least this appears to be the case if one may estimate the phagocytic activity of such elements by the amount of phagocytosed pigment and cellular debris seen in their cytoplasm. It is suggested, therefore, that in ducks the initial rise in peripheral counts is a product of erythropoietic stimulation but that the maintenance of the elevated count may, in part, be the product of a depressed rate of blood destruction. Several observations of our own and those of others seem to bear out such a conclusion.

Anderson and co-workers<sup>20</sup> have found that a high blood-building diet is necessary to the maintenance of cobalt polycythemia in the rat. This fact, considered with our findings, may suggest that cobalt, in the absence of such dietary support, ultimately suppresses erythropoiesis. Frost and associates<sup>21</sup> found cobalt to have what they considered a toxic effect on young dogs, an effect that was partially compensated by the simultaneous feeding of whole liver. In the blood films of our ducks killed on the nineteenth and twenty-eighth experimental days there was evidence of increasing intravascular cellular fragmentation which, in association with the increasing marrow hypoplasia, may indicate a toxic process under way in these animals. If this interpretation is correct, it becomes necessary to assume some depression in rate of blood destruction to account for the maintenance of elevated erythrocyte counts. To support this assumption, there is the observation that at the time the greatest amount of cellular debris occurred in the peripheral blood, there was also the least phagocytosed material seen in the reticulo-endothelial elements, which one might normally expect to be loaded with it. These facts lead one to assume some depression in the blood-destroying activity in the duck as a consequence of continued cobalt administration.

To summarize the hematologic and pathologic data in the ducks given cobalt, it may be said that there occurs a period in the latter part of the experiment in which there is a marked increase in the total number of red blood cells, but the percentage of young cells has decreased and the number of erythroblastic cells in the bone marrow has decreased also. Furthermore, the erythrocytes at this time show cytologic evidence of injury. There also is evidence at this time of decreased phagocytic activity of the cells in the reticulo-endothelial system. There is, therefore, a polycythemia with cytologic and pathologic evidence of injury to the erythroblastic series.

Ducks infected with *Plasmodium lophurae* show a hyperactivity of the cells in the femur marrow and also of those in the extramedullary erythropoietic foci.<sup>18</sup> It has been suggested that this hyperplasia was the response of the birds to the acute and rapid loss of red blood cells produced by the malarial parasite. Similar histologic changes are present in the ducks given cobalt and killed between the third and fourteenth day. In malarial infected ducks there is

an anemia; in cobalt-treated birds there is a polycythemia. In each experiment there has been a stimulation of the hematopoietic tissue. These observations in the cobalt-treated ducks, therefore, apparently support the opinion of one of us (R. H. R.), that certain of the pathologic changes in malaria result from the anemia and are not specific for this disease.

#### CONCLUSIONS

1. Polycythemia has been produced in young ducks by subcutaneous injection of cobaltous chloride in aqueous solution.

2. This polycythemia is accompanied by early marrow hyperplasia and an increase in extramedullary erythropoietic masses in spleen, liver, kidney, and suprarenal.

3. With continued injection of cobaltous chloride, there is evidence of marrow hypoplasia, disappearance of extramedullary erythropoietic foci, and a lowered phagocytic activity of the reticulo-endothelial elements of the spleen and the liver.

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TABLE I

## QUESTIONNAIRE—INTENTIONS OF 500 MINNESOTA PHYSICIANS IN ARMED SERVICES\*

Entering service from internship	68
Will enter practice	1
Want short courses	8
Want residencies	59
Entering service from residency	50
Will practice specialty	6
Want short courses	15
Want residencies	29
Entering service from practice	382
Re-entering general practice	31
Re-entering specialty practice	57
Want short courses	200
Want residencies	87
Remain in government service	7

\*These data were provided through the courtesy of Mr. R. R. Rosell, Secretary, Minnesota State Medical Association, Medical Arts Bldg., St. Paul, Minn.

those in Service who would like to return to civilian life immediately upon the war's end. There is every reason to anticipate that the plotted curve of increasing participation in graduate medical education in the postwar years will be plateaulike rather than sharp peaked, comparable, if you like, to the pulse wave of aortic stenosis rather than to that of aortic regurgitation. It is highly probable that the wave of increase will be sustained for at least ten years, and perhaps indefinitely. Recognizing the obvious cause of the ascending portion of the curve as being due to the men gradually returning from Service, the question may well be asked as to what factors are going to maintain the plateau. One is ever more impressed that the old order changeth, yielding place to new, which in this instance is the crescendo increase of group practice and specialization in smaller communities. There appears to be but little doubt that the general practitioner of the future is going to be steadily more of an internist and less of a surgeon; a family physician and counselor who has been trained in the recognition of disease but who has no illusions about his ability to treat it surgically; one who is at least reasonably competent in medical management and at the same time aware of its limitations. In years gone by the general practitioner, in the truest sense of the term, was essential because of the factors of time and distance. Modern transportation is rapidly bringing these factors to the vanishing point. Excluding the temporary difficulties imposed by war, it will seldom be necessary any longer for a patient to be transported for more than a very brief period in order to have an emergency or complex problem of whatever nature cared for by a thoroughly competent specialist. There is little doubt that this trend will continue to develop after the war. We can look forward to a steady increase in the number of strategically located, well-equipped and well-staffed hospitals in smaller cities. Concrete plans along these lines are being contemplated in several states and, as many of you know, the Commonwealth Foundation has initiated a plan of this type in the South during recent years. All of these hospitals will undoubtedly be small centers of group practice requiring varying numbers of medical and surgical specialists.

Thus it seems evident that we are going to have the demand and supply for a sustained plateau in graduate medical education. This will require increased teaching material and staff. In many of the centers of medical educa-

tion the clinical material will suffice if it is made available in the proper way. Some universities, as, for example, those in Boston, have been able to avail themselves fully of the clinical material in their cities, to the great advantage of medical education, clinical research, and the care of the sick. In many other centers, however, a vast amount of first-class clinical material is not being employed to the greatest good. One additional question regarding available clinical material which looms large at the present time is that of the future relationship of the Veterans' Hospitals to medical education. It is generally understood that between three and four hundred thousand beds will be available for veterans throughout the United States. Obviously, a large number of trained specialists will be needed to provide adequate medical care in these institutions, and this need might well have been added to those factors mentioned a few moments ago which are believed likely to maintain the plateau of increase in graduate medical education. One must ask, however, to what extent a considerable segregation of the national clinical material in the Veterans' Hospitals might impose serious limitations upon the universities and teaching hospitals. I shall risk being thought naive to venture the belief that an affiliation of medical schools and Veterans' Hospitals for the purpose of teaching and research would appear to have many mutual advantages. In the past the Veterans' Administration has undoubtedly had to give thought to objections on the part of some to their being used for this purpose. This objection would be removed, however, if the arrangement were worked out on a purely voluntary basis. It is true that the location of many of the hospitals would preclude their use, but in the Twin Cities area, for example, a 500-bed hospital is near at hand. This hospital would provide excellent clinical material not otherwise available, as, for example, in Tropical Medicine.

In contemplating a program for a much larger number of graduates, it is evident that a broader base must be achieved and that this can be effected, with undoubted advantage to the individual, through greater diversification of training. It is clear that the direction which this pursues should depend to some extent upon whether the individual's goal is one of the practice of medicine, after certification of the Board, or whether, mainly, one of clinical teaching and research. In the former case, the plan should place more emphasis upon clinical medicine, while in the latter, somewhat more time should be devoted to the pre-clinical sciences and to experimental medicine. By this, I do not mean to imply that the individual can decide forthwith that he is going to pursue an academic career in medicine. He may have this plan and he may, indeed, have gauged his talents and abilities properly; on the other hand, it may become apparent after a year or so of exploration that he is better suited to the art and science of the practice of medicine than to the science and art of clinical investigation. Thus it is clear that an allowance of time will be necessary at the outset which should take the form of a period of training of equal value in either direction. With these reflections in mind, a program of the general type given in Table II is under tentative consideration.

It is obvious that each individual rotating through this schedule will not have service in all of the departments mentioned. Further, it is certain that some will drop out before the four years are completed, mainly because of real

TABLE II

<i>First Year</i>	
6 to 9 months' assistant residency in medicine.	
3 to 6 months' service in one or two minor fields: pathology, medical gynecology, clinical laboratory medicine, radiology	
<i>Second and Third Years</i>	
Same plan as in first year plus 6 months (total) as clinical assistant with university preceptor (private practice)	
<i>Fourth Year</i>	
6 to 9 months' residency in medicine	
3 to 6 months in a minor subject, as noted in first-year plan	

or imagined financial stringencies. Let us return to this problem in a few moments. The plan is sufficiently elastic to permit more emphasis on clinical training for those who are clearly suited for the practice of medicine and more on the fundamental sciences and on clinical research for those who show promise in the latter direction. Except for periods spent with the preceptor, the individual will be given teaching responsibility throughout, not only to gauge his aptitude for teaching, but also to provide an additional stimulus to the quest for knowledge. He will also be encouraged from the outset to devote whatever unused time his program may permit to a continuing research on a specific problem in medicine.

It may be noted that the clinical assistantship with a university preceptor is assigned to the second and third years. Where it is evident early that a man is best suited for practice, this period will be deferred until the third year. If the individual shows some promise for teaching and research but is not certain of his inclination, the period of contact with the practicing physician may be provided in the second year in order that he may have an earlier opportunity to make due comparison. A number of advantages are envisioned in such preceptorships, among which a better insight into the physician-private patient relationship may be stressed; that is to say, a superior opportunity of studying the art of medicine as compared with that usually available in the teaching hospital. It is recognized, of course, that difficulties will be encountered unless the greatest care is exercised in the choice of these preceptors and in the arrangements made between them and the university at the outset. It is believed, however, that the selection of a few outstanding men will gradually permit the establishment of a tradition of the utmost value. By means of these preceptorships, plus fellowships in certain hospitals not yet included in the graduate program, it is believed that the Department of Medicine of the University of Minnesota would be able to achieve a threefold expansion of its graduate enrollment.

A four-year program of the type just alluded to at once brings up one of the most important problems of graduate education: namely, the ever-increasing age at which an individual completes his formal training. This problem is the inevitable sequel to the need for more time to impart the expanding knowledge of the sciences. Increasing specialization in medicine has served, here, to prevent the problem from attaining impossible dimensions, but I think we are all aware of the economic and social difficulties often imposed upon the young man intent upon securing graduate instruction but unwilling to wait until he is 30 years of age or older before assuming family responsibilities. Not a few have existed penuriously for several years rather than forego either training or family. What measures might be taken to alleviate this difficulty at least in



part? For one thing, a graduated increase in stipend might be provided, depending upon the size of the individual's family, according to the established and thoroughly satisfactory method in use for government employees in certain countries. In this country we are constantly deploring the relatively low birth rate at the so-called "intelligentsia" level, yet our present salary scale in graduate education certainly counterbalances any propaganda which might encourage graduate students to have families, or larger families. If additional subsidies become available for advanced study after the war, some thought might well be given to the establishment of a sliding scale according to the number of dependents.

Another factor of perhaps more importance to the problem of late age of completion is that of the amount of time spent in primary education. As a nation, we have long been wedded to the concept that everyone, with exception of the occasional infant prodigy, must spend eight years in grade school and four in high school before entering college. One is reminded of the old verse of Persius\*: "He still is moist and yielding clay; now, now, ere he congeal, we must make haste and tirelessly shape him on the sharp wheel."

We are just beginning to realize that the ability of school children and their need of a longer or shorter period of primary schooling before entering college must be treated in part, as a problem in bio-statistics. If 18 is the correct average age for college entrance, it is a statistical certainty that there are many who can enter at 17, quite a few at 16, and a fair number even below this age. Conversely, there are many who are not ready at 18, 19, or 20, and of this group the vast majority are not suited for a college education at all. In the past there has been a strong prejudice against acceleration of the primary education in individual cases because of the fear of psychologic maladjustments due to age disparity. There is an increasing feeling, however, that this difficulty can be avoided by an early and careful selection of groups or units which are advanced through the primary schools at varying rates, according to scholastic ability and other factors. I shall not attempt to discuss the difficulties of selection which such a proposal would entail, except to say that many leading educators now regard this general type of selection as entirely feasible on the basis of objective testing. Clinical investigators and teachers, in particular, ought to be vitally interested in this whole problem.

I must yield to the temptation to digress for a moment longer on the topic of primary education. May I recommend to any of you who have not already read it the charming essay of Montaigne on the "Education of Children," first published in 1580. In this, as translated by Florio, Montaigne says†: "Those which according to our common fashion undertake with one selfe-same lesson, and like maner of education to direct many spirits of divers formes and different humours, it is no marvell if among a multitude of children, they scarce meet with two or three that reap any good fruit by their discipline, or that may come to any perfection." This may be unduly severe for the twentieth century, but it is adequate evidence, nevertheless, that the concept which has just been upheld, that is, recognizing individual variations in intelligence and aptitude and teaching accordingly, is by no means a recent one.

\*From *Selected Essays of Montaigne* translated by Donald Frame, published by Walter J. Black, New York, N. Y., for the Classics Club, 1944.

†Published by the Modern Library, Random House, Inc., New York, N. Y.

The accelerated curriculum which the medical schools of this country have adopted during the period of the war should be reconsidered from the viewpoint just presented. There are some medical students who will perhaps not suffer because of the acceleration and who will thus gain time for graduate education. On the other hand, there are many, probably a majority, who should have more time for study and contemplation and for whom the accelerated curriculum is not well suited. I am fully aware of the difficulty which a double curriculum would impose on the administration and faculty of any medical school. I do not offer any solution of this problem but simply wish to emphasize its importance.

Up to the present, the discussion has related in the main to graduate rather than postgraduate education. The war will really draw the distinction between these two to the vanishing point, mainly because of the difficulty which will certainly be encountered in classifying an individual's medical experience during his period of Service. Some will have gone from their brief internships to an Army or Navy hospital where they will have served for two to three years, often under careful supervision and with excellent clinical material and facilities. This group will have gained considerable experience of real value to their future career and may, therefore, have been considered to have had some of their graduate training. Others, however, will have gained little but a broader experience in living, and will, in fact, have lost much of what medicine they learned during their accelerated medical school curriculum. Undoubtedly, the best thing for this group, initially, would be a further period of internship, and it is to be hoped that this can be arranged in many instances.

In addition to those who went from their internship directly into the Armed Services, there will be a large number of men who had been in practice for from a few months to a number of years. Quite a few of the latter will want "refresher" courses lasting from two to six months, while many of the younger men will be willing to devote a year to postgraduate work before returning to practice. The lack of uniformity in this whole group is bound to make for the greatest difficulties both in policy and organization of courses. How can the most training be given to the individuals who are most in need of it? Obviously, a man who has had but nine months rotating internship preceding his Military Service can go directly into practice if he so elects but, just as obviously, to the probable detriment of medical care. Is this individual to be admitted to refresher courses of from six to eight weeks in length? The difficulty is, that if denied this privilege, some few will simply forego the longer courses which may be offered and enter practice sans refreshment. It is certain that this situation will have to be accepted in some measure as one of the inevitable backward journeys incident to war and that the best we can do is to minimize it by trying to induce all of the younger veterans to spend at least six months in an intensive postgraduate curriculum before entering practice.

The character of the postgraduate curriculum offered in any given center will, of necessity, vary with the available clinical material and extra teaching staff. Government or foundation subsidies of one source or another will be essential with rare exception. An attempt to plan a generally applicable curriculum would appear to have little value, but there are, nevertheless, certain guiding principles which we have accepted in formulating our own plans. The

first is to avoid short, purely didactic refresher courses. Eight weeks of intensive clinical instruction is believed to be the minimum length of course which should be planned, and younger men with little experience should be dissuaded from enrolling in the shorter courses and at the same time encouraged to take longer ones.

According to a tentative plan recently formulated\* at the University of Minnesota, postgraduate instruction in medicine will be divided into two parts, as shown in Table III.

TABLE III  
POSTGRADUATE PLAN

A. One-week survey courses in each of following:		
Infectious disease	Radiology	Surgical diagnosis
Respiratory disease	Dermatology	Proctology
Cardiology	Neurology	Genitourinary diseases
Gastroenterology	Psychiatry	Orthopedics
Liver, biliary tract, and pancreas	Medical gynecology	Physical medicine
Hematology	Obstetrics	Anesthesia
Endocrinology—Metabolism	Pediatrics	Physiology
Pathology	Medical otolaryngology	
	Medical ophthalmology	
	Limited to 30 individuals at one time	
B. Six months to one year as volunteer or junior attending staff man rotating on various services		
	Limited to 10 individuals at one time	

As noted in Section A, there are three units each of eight weeks' duration, or six months in all. It is planned to require registration for at least one eight-week unit, with the understanding that individuals may drop out and return at a later date for another unit if so desired. It is also planned that at least one eight-week unit shall be a prerequisite for the six- to twelve-month schedule given in Section B.

Insofar as possible, the majority of the individual's time in both A and B should be spent at the bedside or in the clinical laboratory. The one-week survey courses will be planned primarily for the practicing physician and will specifically exclude operative techniques. Clinics, demonstrations, ward rounds, conferences, and round table discussions will suitably compose a major part of these courses, whether long or short. Reading of pertinent literature will be suggested. Individuals taking the longer courses may, in part, be assigned to ward services as junior attending physicians, depending, of course, upon their respective qualifications. Visual education will be of the utmost value in supplementing all of these functions, and, in fact, a great service will be done to medical education generally by the foundation or Governmental agency which subsidizes a broad program of assistance to medical schools in preparing their own colored movies and lantern slides of various aspects of disease.

Finally, it would be well if a national clearing house or central committee were to be established now which would at once begin to prepare a list of all available locations for graduate and postgraduate education in American medicine. Quite possibly some committee already existing within the structure

\*In conjunction with Dr. W. A. O'Brien, Director of Postgraduate Medical Education, University of Minnesota.

of a representative organization will undertake this task.\* Such a committee could advise candidates as to the minimum period of training that they should receive on the basis of their prewar and war experience either in general medicine or in some special field. At the same time, an applicant could be informed that a fellowship, house officership, or refresher course, as best fitted for his particular case, might be applied for at a given institution. Centralization along these general lines should be of material aid in providing the largest possible number of places and in accomplishing this end in the best interests of medical care and clinical research.

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\*Since this address was given, a letter has been received from the Council on Medical Education and Hospitals, and the Committee on Postwar Medical Service, of the American Medical Association, indicating that they will act in this capacity.

## Scientific Program

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### CHANGES IN THE PRECORDIAL ELECTROCARDIOGRAM PRODUCED BY EXTENSION OF ANTEROSEPTAL MYOCARDIAL INFARCTION

FRANCIS F. ROSENBAUM, M.D., FRANK N. WILSON, M.D., AND  
FRANKLIN D. JOHNSTON, M.D.  
ANN ARBOR, MICH.

An electrocardiographic study of two patients, each of whom experienced two attacks of severe chest pain of the anginal type within a short period of time, illustrates the value of multiple precordial leads in diagnosis of the extension of an anteroseptal infarct.

In the first case, the limb leads taken six and one-half hours after the onset of symptoms are negative except for slight terminal inversion of the T waves in Leads I and  $V_L$ , changes somewhat suggestive but not diagnostic of recent myocardial infarction. The multiple precordial leads, however, show QRS and T-wave changes in  $V_2$  and sharply inverted T waves in Leads  $V_3$ ,  $V_4$ , and  $V_5$ . Approximately thirty-six hours after the first attack of pain, the patient had a second more severe and prolonged period of substernal distress associated with marked prostration. This attack was accompanied by the appearance of characteristic and progressive QRS and T-wave changes in Leads I,  $V_L$ ,  $V_3$ ,  $V_4$ , and  $V_5$ , indicating a lateral extension of the infarcted zone of muscle.

The second patient was first seen approximately eighteen hours after several sharp attacks of atypical angina pectoris. The electrocardiograms taken at that time display prominent QS deflections and inverted T waves characteristic of myocardial infarction in Leads  $V_1$ ,  $V_2$ , and  $V_E$ . Sharp terminal inversion of the T waves is seen also in Leads I, II,  $V_L$ ,  $V_3$ ,  $V_4$ ,  $V_5$ , and  $V_6$ , but no significant changes in the QRS complexes appear in these leads. Two days after the examination, the patient experienced a prolonged attack of severe chest pain. The electrocardiograms taken three months later show QS or large Q waves and sharply inverted T waves in Leads  $V_2$ ,  $V_3$ ,  $V_4$ , and  $V_5$ , indicating that the zone of infarction had extended laterally.

The observations made illustrate the worth of multiple unipolar precordial leads in the diagnosis of the extension of an anteroseptal infarct. They also suggest that, in patients with coronary arterial disease, at least some of the attacks of pain which are usually considered premonitory or prodromal symptoms of myocardial infarction represent the development of a small infarct and that the more characteristic symptoms of coronary thrombosis which often occur later are due to an extension of the initial lesion. Evidence is again presented that infarcts which are anteroseptal in location, as shown by diagnostic changes in leads from the right precordial area, often fail to produce equally significant changes in the limb leads.

## DISCUSSION

DR. L. N. KATZ, Chicago, Ill.—The evidence presented by the Ann Arbor group would be more convincing if they had obtained control electrocardiograms preceding the recent myocardial infarction. The possibility remains that the patient might have had an old healed infarct to account for the appearance of the first record. There are cases on record in which following severe precordial pain, the electrocardiogram, including chest leads, has been normal or not characteristic. While the results of the authors are suggestive, their argument would be greatly strengthened if the possibility that the first record shown was not due to an old, healed infarct had been established.

DR. DOUGLAS DEEDS, Denver, Colo.—I was interested to note from Doctor Rosenbaum's slides that the central terminal lead from the left arm exhibited inversion of the T wave while the same lead from the right arm and left leg did not. Two years ago it was my privilege to present to this Society electrocardiographic data based upon the comparison of precordial Leads CR, CL, CF, and CT in an increasing series of abnormal cases now numbering in the hundreds. For several years it has been my belief that the best routine precordial lead technique consists of Leads  $CL_2$ ,  $IV_L$ ,  $CL_6$ , and the Whitten amplification of Lead I. Therefore, I was keenly interested in noting the corroborative evidence supplied by Doctor Rosenbaum's slides in which the central terminal lead from the left arm exhibited T-wave inversion, whereas the same lead from the right arm and left leg exhibited upright T waves. Continuing investigation continues to demonstrate that not infrequently the central terminal technique nullifies the valuable "sensitivity" of the CI leads in acute cor pulmonale and in posterior left ventricular infarction and strengthens my belief that the left arm is the best so-called indifferent electrode to use in routine precordial lead electrocardiography.

DR. ROSENBAUM (closing).—I agree with Doctor Katz' suggestion, that if earlier, control electrocardiograms had been available, the observations might be somewhat more impressive. It should be noted, however, that the first patient had no cardiac symptoms whatever prior to those which appeared six and one-half hours before the first standard leads and twenty-seven hours before the precordial leads were taken. This additional slide presents the records of a patient who had a myocardial infarct two or three weeks earlier. Except for slight flattening of the T waves in Lead I, the standard leads and the leads from the left precordium ( $V_4$ ,  $V_5$ , and  $V_6$ ) are well within normal limits. However, the unipolar lead from the left arm ( $V_L$ ) shows sharp terminal inversion of the T waves and  $V_2$  and  $V_3$  (from the right precordium) display QS deflections and inverted T waves characteristic of recent anteroseptal myocardial infarction. Some of the cases of "impending myocardial infarction" which have been reported to show no or minor changes in the electrocardiograms, which have been limited to the standard leads and a single lead from the left precordium (usually  $IVF$ ), may actually have been similar examples of anteroseptal myocardial infarction. Again, I would like to emphasize that we do not feel there is sufficient evidence at the present time to indicate that all premonitory symptoms are associated with infarction. There are probably many instances in which these manifestations are not associated with actual infarction.

In reply to Doctor Deeds' question regarding the T waves in the lead from the left arm ( $V_L$ ), it is true that in both of these patients the T waves were inverted in this lead. This is the usual finding in infarcts which involve the anterior aspects of the left ventricle. However, if the left arm were used as the indifferent point in taking the precordial leads, the effect would be to subtract the inverted T wave of the unipolar left arm lead from the negative T wave of the precordial leads, thereby diminishing rather than increasing the evidence of infarction.

## THE USE OF THE AUGMENTED UNIPOLAR LEFT LEG LEAD IN THE DIFFERENTIATION OF THE NORMAL FROM ABNORMAL Q WAVE IN STANDARD LEAD III

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An attempt was made to evaluate the diagnostic significance of the QRS pattern in the augmented unipolar left leg lead (Lead  $AV_F$ ) as a means of establishing or excluding the diagnosis of posterior infarction. A series of forty-nine patients was selected for study because of the presence of a prominent Q wave in standard Lead III. One additional patient with posterior infarction was included who did not have Q waves but exhibited the classical ST-T wave changes in Lead III.

Multiple precordial and unipolar extremity leads were taken on every patient and esophageal leads were taken on forty-four of the fifty patients. The presence of a posterior infarct was established in a total of twenty-five patients—in four of these by autopsy and in the remaining twenty-one by typical esophageal leads. The infarct was months or years old in all but four patients. Posterior infarction was excluded in a total of twenty-five patients—in three by autopsy and in the remaining twenty-two by negative esophageal leads.

In all cases where posterior infarction was excluded, a prominent Q3 or QS3 was present. This amounted to 25 per cent or more of the tallest R in twenty-three of the twenty-five patients; from an examination of the standard leads alone, many of these cases could not be distinguished from cases proved to have old posterior infarction. The pattern of the QRS in lead  $AV_F$  proved to be of considerable help in this differentiation.

A  $QAV_F$  which was 25 per cent or more of  $RAV_F$  was found in twenty-two of the twenty-five patients proved to have posterior infarct, and in only three of the twenty-five patients in whom the diagnosis of posterior infarction had been excluded. In both patients with posterior infarct who had a Q3 but failed to show  $QAV_F$ , esophageal leads suggested that the infarct was located high on the posterior wall, near the auricular margin. The voltage of the QRS wave in Lead  $AV_F$  was low in two of the three uninfarcted controls who showed a Q/R ratio exceeding 25 per cent in this lead. In the remaining case, the deep Q wave was present in Lead  $AV_F$  when the patient was recumbent, but disappeared when the curve was taken in the erect posture.

### DISCUSSION

DR. EDWARD MASSIE, St. Louis, Mo.—In the Heart Station at Barnes Hospital, we routinely have the patient take a deep breath in Lead III. It has been our impression that, in those patients with normal hearts, the Q3 tends to disappear or at least become significantly smaller following a deep inspiration. I should like to ask for other opinions regarding this point, because certainly this would be a simple way to differentiate a normal from an abnormal Q3.

DR. J. S. LADUE, New Orleans, La.—I would like to ask whether or not any correlation between the anatomic axis of the heart was made in patients who had prominent Q waves in the augmented unipolar leg lead but who failed to show infarction at autopsy. The horizontal position of the heart might account for the enlarged Q.

DR. LOUIS N. KATZ, Chicago, Ill.—The term unipolar lead is misinforming. It still remains to be demonstrated that these leads show only the potential changes of one extremity, and I wish it to go on record that I believe this concept is far from proved.

DR. FRANKLIN D. JOHNSTON, Ann Arbor, Mich.—I think the chest lead is worth while. I would like to point out one further point, however—the importance of potential variation of the left leg in posterior infarction. The left leg, of course, enters into the production of II and III standard leads. Furthermore, the fallacy of the left leg in these two leads is the same. Therefore, if alterations in the potential of the left leg are going to produce a Q wave in Lead III, they will also produce Q waves in Lead II. That particular point of the appearance of Q waves in Lead II as well as in Lead III is of significance. Several years ago Durant made a study of a small group of patients in a heart station and pointed out the importance of having small Q waves in Lead II as well as Q waves in Lead III.

DR. CHESTER M. KURTZ, Madison, Wis.—For several years at the Wisconsin General Hospital we routinely have been taking a strip of Lead III with the breath held in deep inspiration and have found it a very valuable procedure. In general, a prominent Q3 resulting from myocardial infarction or serious coronary sclerosis tends to persist, while a Q3 which is of no clinical significance is very likely to disappear. I do not mean to imply that this procedure can in any way replace the method outlined by Doctor Myers but simply mention it in corroboration of the experience obtained by the group at Barnes Hospital in St. Louis.

DR. MYERS (closing).—Both Doctor Massie and Doctor Kurtz have called attention to the well-known tendency of deep inspiration to obliterate the normal Q3. This did not take place in some of our patients having a normal Q wave in Lead III. Furthermore, respiratory changes in amplitude may occur in the Q3 associated with posterior infarction. Hence, the practice of taking a portion of Lead III during deep inspiration is a helpful but not absolutely conclusive procedure in the differentiation of the normal from the abnormal Q3.

With reference to Doctor LaDue's comment, we are attempting to make a correlation of the electrical axis observed during life with the anatomic axis observed at post mortem. There seems to be a rough correlation but the study has not progressed far enough to justify any positive statement.

Doctor Katz is technically correct in objecting to the use of the term "unipolar" since the potential of the "indifferent" electrode in the Goldberger as well as in the Wilson terminal is not exactly zero. The potential of the "indifferent" electrode, however, is so small it does not detract from the clinical use of Lead AV<sub>F</sub> in the diagnosis of posterior infarction.

Doctor Johnston has brought out the fact that in posterior infarction an abnormal Q wave is often present in Lead II as well as in Lead III. This is certainly true in the acute stage, but as time passes the Q wave tends to disappear from Lead II while it remains in Lead III. In twelve of our twenty-five patients with posterior infarct, Q2 was either absent or less than 25 per cent of the amplitude of R2.



OBSERVATIONS ON THE HUMAN ELECTROCARDIOGRAM DURING  
EXPERIMENTAL DISTENTION OF THE GALL BLADDER

SELIM W. McARTHUR, M.D., AND HOWARD WAKEFIELD, M.D.

(INTRODUCED BY N. C. GILBERT, M.D.)

CHICAGO, ILL.

The association of organic heart disease and biliary tract disease, especially stone-bearing gall bladders, has been emphasized by clinicians for many years. Physiologists have demonstrated the presence of viscerocardiac reflexes, especially in the lower animals, and more recently experimental work on animals has demonstrated reduced coronary blood flow on distention of the stomach and gall bladder. With these ideas in mind, we felt that the study of the distended human gall bladder with the electrocardiographic method might yield further evidence indicating the close relationship between the heart and the gall bladder. It is obvious that the results of such a study are of much importance to the surgeon and internist.

*Procedure.*—In the present series of seven female patients whose ages ranged between 34 and 78 years, all had stone-bearing gall bladders and cholecystectomy was performed on each patient. In the preoperative clinical study, electrocardiograms were made as controls. The preoperative medication usually consisted of  $\frac{1}{6}$  to  $\frac{1}{4}$  gr. of morphine sulfate hypodermically and  $\frac{1}{100}$  gr. of atrophine sulfate or  $\frac{1}{150}$  gr. scopolamine in some cases.

Control electrocardiograms were made with a portable instrument while the patient was still awake on the operating table. Additional tracings were made as the patient was going under and especially during the third plane of anesthesia. Cyclopropane was the usual anesthetic; in some instances a mixture of cyclopropane and ethylene was used. During crucial procedures most observations were made with Lead II. Precordial leads were omitted entirely, because the chest lead might be in the surgeon's way and, what is probably more important, we wished to approach the subject with a more stable lead, so Lead II was our choice. When there was time, some observations were also made with Leads I and III.

After the gall bladder was isolated, about 30 c.c. of normal salt solution were injected suddenly through a fine hypodermic needle into the fundus of the gall bladder. In some cases quite a high degree of pressure was obtained in the gall bladder, especially if the cystic duct was blocked with a stone. Tracings on Lead II were also made of such procedures as cutting the skin, pulling the peritoneum, and traction on the gall bladder and cystic duct. Tracings were made during convalescence.

*Summary.*—

1. During the experimental distention of the human gall bladder, we have observed either an increase in heart rate (four patients) or no change in rate at all (three patients). In our small series, slowing of the heart rate has not been observed. When the heart rate is going to accelerate after distention of the gall bladder, the increase in rate usually occurs immediately. After release of the pressure in the gall bladder, the slowing down of the heart rate is a gradual affair.

2. Depression of T II to the isoelectric level has been observed; complete negativity of T II has not been seen so far.

3. Extrasystoles occur frequently after distention of the gall bladder.

4. Increased P-R interval (partial block) has been observed. The block disappears rather quickly after release of the pressure in the gall bladder (by aspiration of contents).

5. Traction on the gall bladder or cystic duct in some patients speeds up the heart rate almost immediately.

6. In our experience, the heart rate usually slows down during the third plane of anesthesia, before surgery is started, either with cyclopropane or a mixture of cyclopropane and ethylene. There is also a better definition of all the components in the electrocardiogram. We have seen little arrhythmia or conduction disturbances during the third plane of anesthesia in our control tracings.

7. Cutting the skin and dissection of the subcutaneous tissues down to the peritoneum usually produced no changes in the electrocardiogram. In one patient, pulling on the peritoneum speeded up the heart rate.

*Conclusion.*—These observations indicate that it is impossible to predict what will happen in the human electrocardiogram after distention of the gall bladder. There is no specific reaction or pattern in the electrocardiogram which is characteristic of gall bladder distention. In some patients distinct changes occur immediately in the electrocardiogram after distention of the gall bladder with normal salt solution; in others, little or no reaction occurs.

These observations are to be regarded as a preliminary report. Further observations with some modification of technique are being made. The studies will be reported subsequently after a much larger series has been observed. We have been unable to find any similar published reports in the literature in which the human gall bladder has been distended with normal salt solution and studied with the electrocardiographic method.

#### DISCUSSION

DR. JOSEPH A. CAPPS, Chicago, Ill.—These findings are quite interesting. The interpretation of the mechanism by which dilatation of the gall bladder produces heart block is rather puzzling. We might consider as an analogy the effect of traumatizing the inflamed visceral pleura, since the pleura has the same innervation as the gall bladder.

Prolonged irritation of the inflamed visceral pleura often produces slowing of the pulse, and at times actual stoppage of the heart. This is a cardio-inhibiting reflex. So the heart block resulting from dilatation of the gall bladder would suggest a vagal effect.

Commonly enough the pulse rate in the essayists' experiments was not slowed. It may be that the atropine administered preoperatively counteracted the slowing effect of the vagus nerve but did not prevent impulses from passing to the bundles of His, resulting in transient heart block. I would suggest to the authors that further observations be made with the omission of the atropine.

DR. DONALD D. KOZOLL, Chicago, Ill.—I would like to ask the investigators if they attempted distention of the common bile duct with these observations.

DR. G. K. FENN, Chicago, Ill.—I was interested because we reported the results on the coronary circulation with distention of the hollow viscera some

time ago. I would like to ask if the authors noticed any clinical evidence of effect on the coronary circulation.

DR. WAKEFIELD (closing).—I think that Doctor ('apps' point about atropine is a good one. I do not know the answer in fact. I certainly would say that the dose that is used was not great enough,  $1_{100}$  to  $1_{150}$  gr., to paralyze the vagi. Otherwise we would not get any response because the work of Fenn and Gilbert has shown where the vagi are paralyzed in dogs, either by section or paralysis, you do not get any reaction.

Regarding Doctor Kozoll's question about distention of the common duct itself, we have not done that in the operating room. Our immediate problem was the effect of the distention of the gall bladder itself. We distended the common duct in a patient postoperatively in whom Doctor McArthur had left a T tube. However, that is nothing new because Ravdin has made a demonstration of what happens there. We found the same result. We did not have a ballisto-cardiograph and we could not determine the cardiac output as Starr has done, but we did have the patient connected to the electrocardiograph and distended the common duct through the T tube to the point where it produced substernal pain and there was no change in the pulse rate and very little fluctuation in blood pressure and no change in the tracing. I think Ravdin in 1942 before the American Surgical Association spoke of the same thing. We considered that another problem. The results have not been as interesting as what happened in the distention of the gall bladder itself.

Doctor Fenn's question is interesting as to what clinical results we have seen in these patients by getting rid of a stone-bearing gall bladder in which there may have been an anginal syndrome. There have been several dramatic results by removing a stone-bearing gall bladder. The woman in whom we produced the complete heart block by traction on the gall bladder had had angina pectoris for years. One year before surgery she had an anterior infarction and she had this stone-bearing gall bladder. She could hardly walk a block without using five or six nitroglycerine tablets. Her immediate remark postoperatively was that her chest felt better than it had in years. I understand she can walk a block at ordinary pace without any pain. I do not think gall bladder surgery is going to cure all of the patients entirely. I think if the patient referred to should walk fast enough with a full stomach and against a cold head wind she will get the pain. The pain is minimized and she feels better. She is not having any more gall bladder colics.

### GENESIS OF CRUSH SYNDROME\*

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INDIANAPOLIS, IND.

A state which resembles that clinically described as crush syndrome may be produced in rats by intravenous injection of metamyoglobin after release of compression from one crushed hindlimb. This renal injury is apparently of dual origin, arising (a) in renal vasoconstrictive ischemia, which follows skeletal trauma (Corcoran; Taylor, and Page, *Ann. Surg.*, 1943), and (b) in intratubular, possibly intracellular, deposition of metamyoglobin and release of hematin.

The means of treatment suggested by this concept of the genesis of crush syndrome were explored. Intravenous injection of sodium bicarbonate or citrate, elsewhere recommended, was found to be contraindicated. Varying degrees of

\*This work was done under contract with the Committee on Medical Research, Office of Scientific Research and Development.

protection were obtained by (a) injection of diuretics—such as sucrose, manitol, or sodium sulfate, (b) application of a compression bandage to the limb after release of crush, and (c) injection of a colloidal plasma substitute, such as gelatin alone, or, better, in M/6 sodium lactate. The most satisfying results, as shown in the prevention of mortality and of renal functional and structural impairment, followed use of a combined treatment in which a pressure bandage was applied to the crushed limb and gelatin in sodium lactate injected intravenously.

#### DISCUSSION

DR. P. K. KNOEFEL, Louisville, Ky.—The application of a tourniquet would lead to reduction in circulating blood volume which would undoubtedly reduce the local blood flow *per se* and cause vasoconstriction. That might serve as an explanation without introducing any circulatory substance. I would like to ask for an explanation of how that could be ruled out.

QUESTION.—What do you mean by compression bandage?

DR. CORCORAN (closing).—The compression bandage we used was designed by Doctors Taylor and Page. It is of an elastic weave and is permeated with an extensible plastic. It was applied spirally from toes to groin after release of the crushing cords and left in place thereafter.

The basis for its use lies in the fact that the renal ischemia elicitable by partially occluding limb tourniquets is almost entirely prevented by prior application of snug plaster encasements. This ischemia is only very incompletely relieved or prevented by renal denervation or by transfusion of large volumes of blood. Its onset is associated with the appearance in plasma of a substance which causes vasoconstriction of the isolated rabbit's ear. It seems likely that this vasoconstrictor is the major cause of renal ischemia in skeletal trauma. Its release from the site of injury is inhibited by snug plaster encasements: thus, the compression bandage used was intended to prevent renal ischemia and the gelatin-lactate solution to restore plasma and interstitial fluid volume and initiate diuresis. The two etiologic factors of crush syndrome were thus simultaneously attacked.

#### EVALUATION OF PECTIN AND GELATIN SOLUTIONS USED IN THE TREATMENT OF SHOCK: HISTOLOGIC CHANGES PRODUCED IN THE HUMAN BEING

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A comparison of the effects of 257 liters of gelatin given to 162 patients and of 235 liters of pectin given to 155 patients was made from a therapeutic, hemodilution, hematologic, and histologic point of view. Approximately half of the patients were in shock and the others were hospital controls.

Previous work has shown an almost identical effectiveness of similar quantities of both these solutions in the treatment of patients with shock produced by a variety of causes despite a marked difference in chemical nature, concen-

\*At present in service with the Armed Forces.

tration, and physical characteristics. Furthermore, both produced a comparable hemodilution in regard to degree, duration, and a relative leveling off of hemodilution with larger doses. This leveling off (or decreasing hemodilution with increasing amounts of the solution) is more marked in patients with anemia or hypoproteinemia. The hypothesis is suggested that the hemodilution is started by the macromolecular solution but maintained by other substances, possibly mobilizable tissue proteins.

The increase of the sedimentation rate was selected as an example of the hematologic changes. It was of similar degree with both pectin and gelatin and showed the same tendency to level off with higher doses.

The two substances differed decisively in the production of tissue changes as observed from the necropsies of twelve patients given gelatin and ten patients given pectin at varying intervals prior to death and from the liver biopsies of eight patients, four of whom were given pectin and four gelatin prior to operation. Gelatin produced only a transient vacuolization of the proximal convoluted tubules of the kidney similar to that seen after sucrose. In four of ten patients who received rather large amounts of pectin, splenomegaly was produced. Histologically, the deposition of a peculiar material in the reticulo-endothelial cells of the spleen, Kupfer cells of the liver, glomerular tufts of the kidney, and alveolar cells of the lung was prominent. Vacuolization of the epithelium, endothelial cell proliferation, giant cell formation, and hyalinization of renal glomeruli were seen. Although it is difficult to identify this material chemically, it does stain with ruthenium red, a dye used for the staining of pectin. It resembles amyloid in deposition but not in staining reaction. The same picture was reproduced in five rabbits given varying amounts of pectin; rabbits receiving gelatin showed insignificant changes. These findings indicate that pectin is a reticulo-endothelial irritant while gelatin does not produce tissue reaction.

#### DISCUSSION

DR. CHARLEY J. SMYTH, Eloise, Mich.—I think that Doctor Popper's presentation is very important because it shows the limitations and the potential dangers of pectin administered intravenously. About two years ago at the Eloise Hospital, Detroit, Mich., we undertook a study to evaluate the various substances advocated for use in the treatment of patients in shock. Through the courtesy of Doctor Frank Hartman, of the Henry Ford Hospital, we obtained some pectin which was used to study its influence on the plasma volume of normal human subjects. In these investigations Evans' blue dye No. 1824 was used. In each of five patients in whom we injected pectin and the dye a generalized purpuric eruption occurred approximately nine days following the injection. In similar studies using 5 per cent gelatin (Upjohn) in which both gelatin and the dye were used we observed no untoward effects. In addition, we have treated approximately 150 patients with 5 per cent gelatin in an attempt to evaluate its effect in controlling shock in patients undergoing surgical operations, and in none of them have we seen any deleterious reactions which we could attribute to gelatin.

DR. KOZOLL (closing).—I can only confirm what Doctor Smyth said about the eruption. In three patients who received 5,000 c.c. or more of pectin in divided doses, we saw this hemorrhagic macular rash over the extensor surface

of the extremities and no dye was administered. It appeared to us that possibly this occurrence with Evans' dye was coincidental. The patients did not present any hematologic changes and the rash was of no significance.

## CHANGES IN THE TREATMENT OF TOXIC GOITER PRODUCED BY THIOURACIL

W. O. THOMPSON, M.D., AND P. K. THOMPSON, M.D. (BY INVITATION)  
CHICAGO, ILL.

Most of what has been written on the treatment of toxic goiter up to the present time is out of date. Data thus far available appear to warrant the following conclusions:

1. In most patients with toxic goiter, thiouracil, when administered in proper doses, will cause a reduction in basal metabolism to within normal limits and maintain it there indefinitely.

2. In some patients, after the basal metabolism has been maintained at the normal level for six months or more by the administration of thiouracil the drug may be omitted without any increase in basal metabolism occurring.

3. It, therefore, appears probable that in patients who are not hypersensitive to the drug and who do not develop serious toxic reactions, toxic goiter may be treated by the administration of thiouracil alone. It will, of course, take several more years to determine the precise status of the drug with regard to the actual percentage of cures and the presence or absence of untoward late complications.

4. When surgical measures are employed, the drug is of great value in preoperative preparation, particularly in patients who have the disease in a very severe form and cannot be prepared by the various measures commonly employed before the introduction of thiouracil. In nearly all such patients who are not hypersensitive to the drug long periods of hospitalization before operation may be avoided by its administration.

## UNUSUAL REACTIONS FOLLOWING THE USE OF THIOURACIL

SAMUEL F. HAINES, M.D., AND F. RAYMOND KEATING, JR., M.D. (BY INVITATION)  
ROCHESTER, MINN.

This report deals with two patients with severe recurrent exophthalmic goiter who were treated with thiouracil. In both instances the thyroid gland was not palpably enlarged but symptoms of hyperthyroidism were severe and the basal metabolic rate was greatly elevated. In each case the administration of thiouracil was followed after several days by diminution of the signs and symptoms of hyperthyroidism and by lowering of the basal metabolic rate. Transient painful swellings of subcutaneous tissues occurred; at the same time, the value for the plasma chloride was increased and the carbon dioxide combining power of the plasma was decreased. In one patient, myoclonic contractions of various muscles, more particularly of the facial muscles, occurred, and at the same time severe somnolence and confusion were present. The myoclonic twitchings persisted for several days after the administration of thiouracil was stopped. Sub-

sequent administration of the drug on two separate occasions was followed rapidly by a resumption of the untoward symptoms and signs. In the other patient, a similar state of somnolence and mental confusion occurred during a second period of administration of the drug. It is felt that these reactions probably were dependent upon a toxic disturbance of the central nervous system.

Since the presentation of this paper, a reaction similar to the foregoing has been produced in one of these patients by the administration of an inert substance. This raises a question as to the exact cause of the previous reactions in both patients. Further study in an attempt to determine more of the nature of the reactions is being carried out.

#### DISCUSSION

DR. M. A. BLANKENHORN, Cincinnati, Ohio.—I would like to ask Doctor Thompson to elaborate a little more on the management of the patients in addition to the drug therapy. In the older days when surgical and medical methods were compared, it was often shown that the circumstances under which the patient had to live during the period of medical management had a psychologically bad effect while that with surgery was psychologically good. Would Doctor Thompson describe the other circumstances?

DR. C. C. STURGIS, Ann Arbor, Mich.—This treatment has been instituted in all patients with toxic goiter on the medical wards of the University of Michigan Hospital. To date, about thirty patients have been observed and followed long enough to judge the immediate results. Without exception all have had a decrease in the basal metabolic rate and other gratifying evidences of improvement. My experience, therefore, agrees that its therapeutic effects are most promising.

Three of our patients had a leucopenia and an additional one a febrile reaction. Associated symptoms, when present, were mild, and in all instances the drug was resumed without incident after it had been discontinued for a few days. Tentatively, we believe that untoward symptoms are most likely to occur soon after the treatment is begun, but they may appear after the therapy has been given for a long time.

Some surgeons complain that following thiouracil therapy the thyroid gland develops increased vascularity which causes excessive bleeding if the patient is operated upon. This may be averted, however, if iodine is also given for two weeks prior to the operation. Occasionally, malignancy may develop in a patient with a toxic adenoma during the course of treatment, as it did in one of our patients.

DR. MARTIN GOLDNER, Chicago, Ill.—My observations at the University of Chicago are based on a series of about twelve patients. Those were patients that had refused operation and had undergone a relapse. All those had proved to be iodine-resistant. On the basis of these observations we can confirm Doctor Thompson's observations on the effect of the drug. In our uncomplicated cases we found that one month, two months, or three months of treatment is necessary. Also we have seen a high percentage of toxic complications—I would say in about 30 per cent of our patients, which is quite a number in a small group. Three or four showed toxic symptoms. There was a severe toxic psychosis in one patient. After treatment of about one month in the dosage recommended, there were no organic findings and no changes in the blood count. The second patient was regarded as already toxic with a generalized adenopathy. This patient was treated with the same dose for three weeks and developed fever and generalized swelling of the lymph glands around the neck. The drug was discontinued. In three days the high temperature decreased and the swelling of the glands disappeared. After one week the same dose was repeated and

the same symptoms occurred. An interesting observation was that patients who proved to be iodine-resistant after thiouracil treatment responded to iodine.

DR. G. T. EVANS, Minneapolis, Minn.—I would like to report on a series of twenty-six cases at the University of Minnesota Hospitals. For the most part, the results have been very gratifying. However, five toxic reactions were observed. One was a severe rash. In another patient with marked leucopenia we saw the neutrophils go from over 1,000 to under 100 in a two-day period; although the drug was stopped, this lasted into the fourth day thereafter. Unless counts are made frequently, reactions like this will not be caught in time. A toxic manifestation which has not received much attention is spontaneous purpura. This occurred in one patient. In two subsequent patients the cuff test was routinely done and became markedly positive under thiouracil therapy. We believe that observations of bleeding tendency will need attention in the administration of this drug.

DR. PAUL H. NOTH, Detroit, Mich.—We noticed severe cerebral toxic manifestations followed by exitus apparently due to thiourea in a Negro man 40 years of age who suffered from a nodular goiter with associated thyrotoxic heart disease. The cardiac failure was controlled by rest and digitalis. Variable glycosuria and fasting blood sugar levels ranging between 118 and 148 mg./100 c.c. were attributed tentatively to the hyperthyroidism. On the twenty-fifth day following admission thiourea was administered orally in a dose of 1 Gm. every six hours. Five days later the patient appeared weak and complained of dizziness. The next day he vomited on one occasion. That evening he became irrational, restless, semicomatose, and incontinent. Respirations were shallow and rapid. The thiourea was discontinued. The level of blood sugar increased to 396 mg./100 c.c. and the blood urea to 79 mg./100 c.c., but the  $\text{CO}_2$  combining power showed only a moderate reduction to 36 vol. per cent. Leucocytes of the blood numbered 13,800. The lack of clinical and laboratory evidences of diabetic acidosis excluded this as the cause of his symptoms. Therapy consisted of the usual measures for control of diabetes. The blood sugar level fell slightly and the  $\text{CO}_2$  combining power remained at about the same level. The patient's mental state did not change and he died two days later.

Another patient with hyperthyroidism and diabetes showed an otherwise unexplained rise of blood sugar following administration of thiourea.

DR. W. H. BUNN, Youngstown, Ohio.—We have twenty patients who have taken thiouracil. Our experience has been very much the same as those cited. We have had one patient who developed severe jaundice. The pharmaceutical house that sent us our supply of thiouracil told us they had had other cases of jaundice reported.

Our plan has been to stop iodine while thiouracil is being given because it seems to slow its effect. For the surgeon's benefit we give iodine for ten days before operation in fairly large doses. The gland "firms" up and there is no trouble from excessive bleeding.

DR. W. O. THOMPSON, Chicago, Ill.—So far as the psychologic factors in the production of toxic goiter are concerned, it seems to me these are not very important in most instances and have been greatly overdone. Certainly the disease is not psychogenic in origin. There may be occasional instances in which some unusual psychic trauma seems to set off the disease. By and large, however, it is a chemical disturbance. Psychiatrists have developed a great variety of peculiar explanations for many diseased states. After reading some of these explanations one is almost forced to the conclusion that they belong in the realm of stargazing. As our knowledge increases we must conclude that most diseased states can be explained by organic or chemical alterations in the body. Please do not misunderstand me; I am not claiming that all psychiatrists are star-



gazers, but in the past there has been some tendency in this direction. I am very happy to see that one of the most important recent advances in the field of psychiatry is an effort to correlate mental and emotional changes with organic and chemical changes in the central and sympathetic nervous systems.

The pulse rate follows the basal metabolism and actually shows the same change that it does following a reduction in metabolism from any other cause. So far as we can tell, when the metabolism has been reduced by thiouracil administration, the clinical condition of the patient is exactly the same as when the basal metabolism has been restored to normal by removal of the gland.

One word about toxic reactions. This, of course, is a drug not to be used indiscriminately; it is still in the experimental stage, and it is still not available commercially. It will take two or three years more to determine its precise status. Some patients have a definite sensitivity to the drug. One woman, following a single dose of 0.1 Gm., showed an increase in temperature to 103° F. with pain in the legs, diarrhea, nausea, and vomiting. That phenomenon was repeated twice. If we had persisted in the administration of the drug to that patient, the result might have been fatal. It is wise to give an initial dose of 0.1 Gm. to see if there is any sensitivity. The patient afterward must be followed with great care. White counts must be taken at frequent intervals. In spite of all these toxic reactions, it seems to me that the introduction of thiouracil represents a very great step forward and provides us with an important clue as to what is going on in toxic goiter.

DR. KEATING (closing).—We have been impressed with the fact that toxic reactions may take place at any time. We have observed them as early as twelve days after treatment with thiouracil has been begun and encountered a severe toxic rash and fever in one patient who had been taking thiouracil for five and one-half months. The fact that several months have passed does not permit the doctor to relax his caution.

A point which has not been emphasized is the possibility of postoperative crises in patients inadequately prepared for surgery with thiouracil. We have not encountered any, but such reactions have been mentioned by some workers. We feel there is a serious risk in assuming that thiouracil and iodine act identically in the preparation of patients for surgery. Patients with exophthalmic goiter who have been given iodine may sometimes be operated on with comparative safety while the basal metabolic rate is still elevated. Until more is known about it, I feel that it is hazardous to assume that this is the case in patients prepared for surgery with thiouracil.

#### THE UPPER LIMITS OF HEAT AND HUMIDITY TOLERATED BY ACCLIMATIZED MEN WORKING IN HOT ENVIRONMENTS\*

MAJOR WILLIAM B. BEAN, MAJOR LUDWIG W. EICHNA (BY INVITATION),  
AND MAJOR WILLIAM F. ASHE (BY INVITATION)  
MEDICAL CORPS, ARMY OF THE UNITED STATES

Defining the environmental ceiling for work in the heat is a complex problem. One must control state of acclimatization, nature of environment, work, fitness, and morale of the subjects, their clothing, diet, and salt and water intake. The condition of the men during and after work and its "cost" to the subject in terms of physiologic disturbances indicate the upper limits. Because of many possible combinations of heat, humidity, wind, radiation, work, and clothing, an arbitrary set of conditions was chosen for investigation. Thirteen healthy young garrison troops were first acclimatized to dry and moist heat. They then

\*From the Armored Medical Research Laboratory, Fort Knox, Ky.

worked in fourteen environments approaching and exceeding tolerable environmental limits for work. The environments varied only in dry and wet bulb temperatures; no radiant energy was supplied. Air movement was that created by the unclothed men walking single file at three miles per hour around the laboratory hot room on a seventy-seven foot track, carrying a twenty-pound pack. The work consisted of walking continuously for four hours. The energy expenditure was approximately 300 calories per hour. Water salted to 0.1 per cent was given when requested and replacement of that lost as sweat was urged. Data were obtained for various combinations of D.B.T.\* between 93 and 121° F. and W.B.T.† 90 and 96° F. and the conclusions apply only to this range of environments.

The W.B.T. is the important limiting factor determining the ability of men to work in hot environments. The D.B.T. has a minor influence. The limiting W.B.T. is approximately 2° F. higher at a D.B.T. of 100° F. than at 120° F.

Near the upper limits, the range of W.B.T. from an environment in which men work with "relative ease" to one in which work is "impossible," is very narrow: 4 to 5° F. for a given D.B.T.

Below a W.B.T. of 91° F. men work easily and efficiently.

Between W.B.T. of 91° and 94° F. men can work but with difficulty, loss of vigor and alertness, and undesirable physiologic changes.

At W.B.T. of 94° F. and higher, less than an hour of sustained work is possible; acute disability with marked physiologic changes occurs.

Near the upper limits sweating averaged 2.5 liters per hour and reached 3.5 liters in some men.

Undesirable physiologic changes encountered near the upper limits include: (1) Heart rates of 150 to 180; (2) rectal temperatures of 102 to 103.5° F.; (3) average (weighted) skin temperatures approaching 101° F.; and (4) violent and protracted headache, nausea, copious vomiting, abdominal cramps, severe weakness, vertigo, dyspnea, paresthesias, stumbling, disorientation, and collapse with syncope.

## METABOLIC ACCLIMATIZATION TO TROPICAL HEAT

C. A. MILLS, M.D.  
CINCINNATI, OHIO

With the large military forces now seeing enforced activity in regions of tropical moist heat, it is especially important that most careful consideration be given to the effects such heat may exert. Unfortunately, several recent articles from investigators engaged in government-sponsored research projects have presented an incomplete and erroneous picture of what may be expected in such acclimatization. While *vasomotor* adaptation to hot environments is usually complete after three to five days of exposure, all studies have tended to indicate that *metabolic* acclimatization takes place much more slowly—that it begins late in the second week of continuous exposure and is largely accomplished by the end of the third week.

\*D.B.T., Dry bulb temperature.

†W.B.T., Wet bulb temperature.

Previously published and new evidence will be presented to prove the existence of this two- to three-week lag in metabolic adaptation and to deal particularly with the question of heightened thiamine and choline needs in tropical heat. New studies have shown that the heightened need for these two factors continues even when the diets contain 0.5 per cent sulfaguanidine; hence, heat depression of intestinal synthesis cannot account for the findings.

The heightened need in tropical heat is most emphatic for choline. Animals on choline-free diets in the heat and cold show no difference during the first week, and during the first half of the second week acute hemorrhagic nephritis causes an equal number of deaths in the two environments. After the second week, however, growth in the cold goes on practically as well without as with choline in the diet, while in the heat, growth is sharply affected.

Johnson, Taylor, Keys, and Holt have claimed that animal findings fail to hold for man and that human tropical needs are not different from those of temperate climates. However, all their published data were based upon only a few hours to a few days of heat exposure and provide no just basis for any deductions whatever regarding nutritional needs in the continuous moist heat of tropical lowlands. Human studies along this line must take into account the two- to three-week lag in metabolic acclimatization.

### THE NEUROVASCULAR SYNDROME PRODUCED BY HYPERABDUCTION OF THE ARMS

THE IMMEDIATE CHANGES PRODUCED IN 150 NORMAL CONTROLS AND THE EFFECTS  
IN CERTAIN INDIVIDUALS OF PROLONGED HYPERABDUCTION AS IN SLEEPING

COLONEL IRVING S. WRIGHT\*

MEDICAL CORPS, ARMY OF THE UNITED STATES

(INTRODUCED BY THEODORE R. VAN DELLEN, M.D.)

Patients have been observed who have developed numbness, paresthesias, trophic changes, and even gangrene of the tips of the fingers as a result of prolonged hyperabduction of the arms. In these patients, the hyperabduction was produced by sleeping or working with the arms in that position. Studies ruled out Raynaud's syndrome, thromboangiitis obliterans, intrinsic and extrinsic tumor of the cervical cord, ruptured nucleus pulposus in the cervical area, infectious polyneuritis, ulnar and median nerve injury, cervical rib and scalenus anticus syndrome.

It was demonstrated that hyperabduction of the arms in the position described resulted in obliteration of the arterial pulse. The question was raised as to whether this constituted a normal or an abnormal finding. Investigation in 150 young adults revealed that obliteration of the pulse could be produced in each arm in approximately 83 per cent of individuals. This could be easily demonstrated in 63 per cent.

The mechanisms playing the most important part are believed to be the stretching of the brachial plexus and the subclavian axillary vessels under the coracoid process with some degree of pinching produced by tightening of the pectoralis minor muscle, and pinching of the vessels and nerves between the

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clavicle and the first rib. Either or a combination of both mechanisms may produce this syndrome. These are not abnormal but normal anatomic arrangements occurring in the majority of individuals but capable of producing a pathologic syndrome after prolonged hyperabduction of the arms. It is to be clearly differentiated from the cervical rib and scalenus anticus syndrome.

#### DISCUSSION

LIEUTENANT COLONEL FORD HICK, Galesburg, Ill.—I would like to ask whether there was any venous compression.

DR. M. A. BLANKENHORN, Cincinnati, Ohio.—T. Wingate Todd, late Professor of Anatomy at Western Reserve University, produced gangrene of the fingers by tying his arm to the bedpost. Since then none of his colleagues and students has reported that this condition occurs spontaneously. I have not seen it. I wonder whether there was something in the matter of soldiers' training or sleeping that predisposed to this.

COLONEL WRIGHT (closing).—In answer to the first question, the venous compression in a few individuals is quite pronounced; in most it is not an important feature. We find certain individuals in whom the pulse cannot be occluded by this position but who develop paresthesias as a result of pinching of the nerves. In other individuals the obliteration of the pulse seems to be the predominant factor. The veins seem to be able to compensate and to maintain the ability to drain the extremity of blood. There are adequate collateral veins for that purpose, especially with the arterial flow so markedly reduced.

I am very much interested in the observations of Todd. We have been searching the literature for further information in reference to this syndrome and have found very little. The first person to develop gangrene in our series was a civilian who started to sleep with the hands in an hyperabducted position two months before the onset of symptoms. He slept very heavily and never associated his symptoms with sleeping. He went on to the point where he developed gangrene of the tips of the fingers bilaterally. He failed to respond to the usual forms of therapy until the relation to posture was called to his attention. By tying the arms loosely to the bedpost at the foot of the bed he changed his sleeping position and made a rather prompt recovery.

The answer relative to soldiers is very interesting. There are many soldiers who reach a point of exhaustion the like of which they have never encountered in civil life. I have been observing these boys for two years. They come in, throw themselves down in an exhausted state, some with their arms hyperabducted, in which position there is enough pinching to produce paresthesias. If they prolong the pinching the paresthesias may persist the next day. Over a period of time this produces an accumulative effect. Teaching them to sleep with their arms down has freed them of symptomatology but in some instances it has taken as long as three or four weeks.

## CARCINOMA OF THE LUNG WITH NONMALIGNANT PLEURAL EFFUSION: RECOVERY BY PNEUMONECTOMY

ALFRED GOLDMAN, M.D.  
St. Louis, Mo

The presence of a pleural effusion in association with a bronchiogenic carcinoma is usually considered as a contraindication to successful operation. Tumor cells can be demonstrated in malignant fluids in approximately 50 to 60 per cent of the cases by the section sediment method of Mandelbaum. It is usually assumed, therefore, that even though these cells cannot be demonstrated, such pleural effusions are cancerous and the patient is therefore doomed. That large nonmalignant pleural effusions may occur with carcinoma of the lung is exemplified by the following case.

The patient, a man 50 years of age, had a bronchiogenic carcinoma of the left lung associated with a large left serosanguinous pleural effusion. Study of the fluid showed no tumor cells, but the presence of a moderate number of polymorphonuclears suggested that a secondary inflammatory lesion was probably present. Pneumonectomy was successfully performed by Doctor J. L. Mudd. The lung showed no sign of pleural involvement. The patient shows no evidence of recurrence of the tumor after eighteen months.

## THE ASSOCIATION OF PERIARTERITIS NODOSA, BRONCHIAL ASTHMA, AND HYPEREOSINOPHILIA

KEITH S. WILSON, M.D. (By Invitation), AND H. L. ALEXANDER, M.D.  
St. Louis, Mo

The triad of periarteritis nodosa, bronchial asthma, and hypereosinophilia was encountered sufficiently often to invite inquiry. Rackemann and Greene in 1939 assembled 237 references to periarteritis nodosa and found twenty-seven cases of bronchial asthma—most of which were accompanied by a hypereosinophilia. We reviewed 300 consecutive cases of periarteritis nodosa proved by tissue section, beginning with the first reported instance of associated asthma in 1914. It is of interest that during the four years of 1940 through 1943, over 150 references to periarteritis nodosa were found with twenty-seven cases of asthma—exactly the same number as in Rackemann and Greene's report that embraced the previous seventy-three years. Thus, there were fifty-four instances of bronchial asthma and periarteritis nodosa in 300 cases, an incidence of 18 per cent. This is doubtless an underestimate since several typical cases were encountered but not proved by tissue section.

Of the fifty-four patients with asthma, the onset of paroxysms antedated the symptoms of periarteritis in all but three. However, the onset of asthma was much later than is usual in that less than 15 per cent began their attacks during the first two decades, whereas some 70 per cent of patients with asthma have their first symptoms before the age of 21 years. In but few cases could extrinsic allergens as foods, pollens, etc., be identified as etiologic factors. As a rule, paroxysms were severe. These facts identify most cases as nonatopic or "intrinsic asthma" as distinguished from the atopic type in which an immunologic mechanism can be identified.

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Dr. WILSON (closing).—Lt. Col. Johnson McGuire's remarks on hyper-eosinophilia and asthma are very interesting. The nature and significance of the eosinophilia is unknown.

The cases of periarteritis nodosa that we studied in the literature were selected because they were proved in practically all instances by biopsy or post-mortem findings. Usually biopsy is necessary to clinch the diagnosis of periarteritis nodosa in the living patient.

The patients, needless to say, were critically examined to be sure the asthma was not due to cardiac conditions such as hypertension. We did not analyze the cases for the incidence of hypertension. In a disease that affects the arterial system so extensively, high incidence of hypertension would not be surprising. Elevated blood pressure is found in the patients in whom the renal vessels are extensively damaged. Rackemann and Greene\* make no mention of the blood pressure in their eight patients. Harkavy†, who is interested in cardiac manifestations as well as allergy, gives the blood pressure as normal or does not mention it.

It is to be emphasized that the true cause of periarteritis nodosa is not known. Hypersensitivity is offered as one of the mechanisms of its production because of the rather high incidence of allergic phenomena in the cases found in the literature. The triad described is only one syndrome of a disease that produces widespread tissue reaction and vascular damage. The diagnosis in many cases is still dependent on evidences of vascular damage and typical biopsy findings. Nevertheless, the presence of allergic phenomena may be a further clue.

We wish to emphasize the fact, as stated by Dr. Watson, that Henning and Kimball quite a while ago emphasized that repeated differential counts must be made lest the eosinophiles be missed. Lebowitz and Hunt said that an abnormal count of eosinophiles may not come until late in the course of the disease. In other words, the eosinophiles are released in the blood stream and then go to the areas affected. This release may be late. In three of the forty-seven patients with asthma and periarteritis nodosa only one count was recorded. One count had 6 per cent eosinophilia and the other two counts had 4 per cent eosinophilia. But, as stated before, these counts could have been higher during some time in the course of the disease.

Dr. M. A. BLANKENHORN, Cincinnati, O.—I was much interested in the last question which was asked and not answered; namely, the incidence of hypertension.

Dr. WILSON.—Our paper did not take up the incidence of hypertension in the analysis of these cases. I thought I made that point clear.

#### METABOLIC EFFECTS OF PARATHYROID HORMONE, DIHYDROTACHYSTEROL, AND CALCIFEROL IN A CASE OF PSEUDOHYPOPARATHYROIDISM

RANDALL G. SPRAGUE, M.D., SAMUEL F. HAINES, M.D., AND MARSCHELLE H. POWER, Ph.D. (By Invitation)  
ROCHESTER, MINN.

A prolonged study of the effects of parathyroid hormone, dihydrotachysterol, and calciferol on calcium, phosphorus, and nitrogen balance was carried out on a patient who presented the clinical features of a condition which Albright has designated as pseudohypoparathyroidism. The metabolic disturbance

\*Trans. A. Am. Physicians 54: 112, 1939.

†J. Allergy 14: 507, 1943.

Blood counts were available in forty-seven patients with asthma. Of these, forty-four had a hypereosinophilia ranging from 11 to 84 per cent with an average of 53.5 per cent. This value is far higher than is observed in uncomplicated bronchial asthma. As controls of 151 cases of periarteritis nodosa without asthma in which counts were available, there were nine instances of hypereosinophilia. The average eosinophile count for the group of 151 cases was 2.5 per cent.

It is believed that the association of severe bronchial asthma and marked eosinophilia in an adult should lead to the strong suspicion of underlying periarteritis nodosa.

Present knowledge of the association of periarteritis nodosa with various forms of allergy are commented upon briefly.

#### DISCUSSION

LIEUTENANT COLONEL JOHNSON MCGUIRE, Cincinnati, Ohio.—At the Cushing General Hospital near Boston in the past year we saw two extremely interesting cases that in many ways simulate these cases and present a relatively new syndrome. These were soldiers that had come from the Southwest Pacific. They had white counts of 65,000 and 85,000, respectively, with 80 per cent eosinophiles in both instances, and severe asthma. At this time, fortunately for the patients, we had encountered a paper by Kenneth Emerson of the Navy in which this syndrome was described as a tropical eosinophilia and the remedy proposed the administration of arsenic. Muscle biopsies eliminated periarteritis nodosa. There were no other signs of this disorder. There were no parasites present in the stools on repeated examination. Both cases lasted six months and both originated in the same island. In both cases carbarsone was given in small doses for a period of ten days, discontinued for a week and a second course administered. Both cases became free of asthma. The blood count became normal, and they were returned to duty. Twenty similar cases were reported prior to Emerson's publication (*Lancet*, January, 1943).

COLONEL E. V. ALLEN, Rochester, Minn.—Will Doctor Wilson discuss the method of making a diagnosis in these cases?

LIEUTENANT COLONEL HAROLD C. LUETH, Evanston, Ill.—At the Research and Educational Hospital we had a patient with intractable asthma in whom a diagnosis of periarteritis nodosa was made. This patient had a marked allergy and was extremely sensitive to many substances.

DR. C. J. WATSON, Minneapolis, Minn.—Hypereosinophilia may disappear suddenly in these patients. We have just recently seen a case exactly of this type, in which the leucocyte count was 65,000 with 60 to 80 per cent eosinophiles. There was epigastric pain such as we see in carcinoma of the pancreas, which was relieved by leaning forward. The diagnosis rested between Hodgkin's disease and periarteritis nodosa. The first biopsy was negative. The second one clearly revealed periarteritis nodosa. After two months this patient suffered what we thought was a thrombosis with perforation, and free gas under the diaphragm, which he survived temporarily. Shortly after the onset of this acute attack his eosinophilia completely disappeared and never recurred. His leucocyte count dropped to 12,000 with 1 per cent eosinophiles. If we had seen the patient during this latter period I am sure we would never have thought of periarteritis nodosa.

QUESTION.—I would like to ask what the incidence of hypertension was in this series of cases.



often much lower and is not so easily restored to normal. If control is not sacrificed, higher protein intake improves the protein pattern and in some cases coincidentally tends to prevent hemorrhages.

Alterations in plasma proteins were studied in fifteen rabbits with alloxan diabetes. Eight animals made diabetic with one or two large injections of alloxan maintained normal plasma proteins, and none developed retinal hemorrhage. Five similarly treated animals developed hemorrhages. Two animals that received repeated small injections of alloxan also developed hemorrhages. The animals showing a reduction in total protein and plasma albumin were more apt to develop retinal hemorrhages. The hemorrhages usually appeared within one to three months. The animal observed for the longest time had hemorrhages with associated reduction in plasma albumin on two occasions; a partial recovery in the percentage of albumin was observed after each hemorrhage. After experimental reduction in plasma albumin and subsequent administration of alloxan retinal hemorrhages appeared in from five to seven days. Retinal hemorrhages occurred in one animal in spite of insulin and polyvitamin therapy.

Lens changes were observed in all animals. The most common type of lens change noted was peripheral lens vacuoles. In general, these became more numerous and encroached upon the central area of the lens. The lens changes progressed in direct proportion to the duration of the diabetes. The progression occurred in spite of insulin and vitamin therapy, but the rate of change was retarded.

#### DISCUSSION

DR. H. T. RICKETTS, Chicago, Ill.—I would like to ask whether retinal hemorrhages were found in the animals deprived of albumin without the production of diabetes. Is it necessary to have an insulin deficiency for these lesions to develop? Second (you may have dwelt on this point but I did not get it if you did), are you able to prevent the development of hemorrhages by feeding the animals a high protein diet before alloxan is administered?

DR. C. J. WATSON, Minneapolis, Minn.—If I understand it correctly, it is your belief that the changes are related principally, but not solely, to low serum albumin. Would you care to comment on the relative infrequency of diabetic retinitis-like changes in conditions such as chronic lipoid nephrosis? I do not believe I have seen changes of the type associated with diabetic retinitis in these cases, even though the serum albumin was at very low levels for long periods.

DR. CECIL STRIKER, Cincinnati, Ohio.—I would like to ask if you can correlate the clinical observations of a very severe diabetes in a juvenile who is notoriously devoid of retinal changes and a relatively mild diabetes in a subject past 50 who is notoriously the victim of retinal changes. One is a mild, very easily controlled diabetic with very severe retinal and lens changes, and the other is a very severe diabetic difficult to control with very little retinal or lens changes.

DR. SCHNEIDER (closing).—In answer to Doctor Ricketts, we believe that diabetes must be present first, at least it must be present in addition to a low albumin. We are wondering ourselves whether a high protein diet will prevent these changes. That sort of investigation is going on at the present time. It is interesting in that same connection that two dogs that had been on a high protein diet had no treatment whatsoever and never developed hemorrhage. The rabbits had been on a much lower protein diet than commonly employed in investigation, which may have made them more susceptible to retinal hemorrhage.

Concerning Doctor Watson's comment, I think the answer to that question is that diabetes must be present. We have gone over cases of liver disease, but such individuals do not have retinal hemorrhage.

Doctor Striker, I do not think I can answer your question. I had hoped someone here might have been able to supply that answer.

## A TENTATIVE TEST FOR THE DIAGNOSIS OF PHEOCHROMOCYTOMA

GRACE M. ROTH, PH.D., AND WALTER F. KVALE, M.D. (BY INVITATION)  
ROCHESTER, MINN.

Although paroxysmal hypertension attributable to pheochromocytoma has become a well-recognized clinical entity, its differentiation from coronary occlusion, hyperthyroidism, histaminic cephalgia, migraine, anxiety states, and persistent hypertension may be difficult. Since none of the known methods of inducing attacks is dependable and since opportunity to observe the patient in a spontaneous attack may not present itself, a simple procedure that would induce attacks at will in these cases would be of great help in diagnosis. The response of fifty-one persons to intravenous injection of 0.05 mg. of histamine base suggested that this method might be used as a clinical test.

Four groups of persons were studied: in Group 1 were nine normal subjects; in Group 2, twenty-two hyperreactors to the cold pressor test; in Group 3, sixteen patients with well-established hypertension; in Group 4, four patients who were suspected of having pheochromocytomas.

In Groups 1 and 3 flushing of face and subsequent headache followed intravenous injection of histamine. These symptoms were mild and disappeared in from five to ten minutes except in one instance in which typical histaminic cephalgia developed. The average elevation of blood pressure was identical with that during the cold pressor test in Group 1 and was 12 mm. of mercury systolic and 15 mm. diastolic less than that during the cold pressor test in Group 2. In Group 3 circumoral pallor appeared often and headaches frequently were severer and lasted longer. The average rise of blood pressure was 6 mm. systolic and 9 mm. diastolic less than that during the cold pressor test.

Three of the four patients in Group 4 had attacks within two minutes after injection. These were characterized by severe headache, distress throughout the abdomen, and later in the chest, groaning, refusal to stay on the bed, and circumoral pallor with subsequent excessive sweating. The systolic blood pressure rose from levels of 110 to 132 mm. to levels of 240 to 260+ mm. while the diastolic pressure rose from levels of 68 to 85 mm. to levels of 142 to 146 mm. In one case these heights were attained after injection of only 0.025 mg. of histamine base. The cold pressor, insulin, and epinephrine tests did not induce attacks in these three patients. At operation, pheochromocytoma was found in the adrenal medulla of each of the three patients. After operation, when the histamine test was repeated, response was normal. The fourth patient in Group 4 responded normally to histamine and operation did not disclose a tumor.

Although the number of cases studied is small, the intravenous injection of small quantities of histamine base may be considered tentatively as a clinical

test in distinguishing the syndrome of pheochromocytoma from other clinical conditions.

## DISCUSSION

WALTER F. KVALE, Rochester, Minn.—Up to the present time we have carried out this test in forty-two patients in whom it was considered necessary to differentiate paroxysmal hypertension as the result of a pheochromocytoma. The test was considered negative in all patients and in four of these in whom an opportunity was presented for exploration of the adrenal regions, no tumor was found.

During the past seventeen months we have had the opportunity of observing three patients in whom a correct clinical diagnosis of pheochromocytoma was made. The rapid injection of small amounts of histamine in each of these three patients produced an attack identical with the spontaneous attacks of which the patients complained and afforded us the opportunity of observing them in their attacks at such times as we wished. An attack was induced at will five times in one patient, twice in another, and once in a third. In each attack there was a marked rise of both the systolic and diastolic blood pressure which promptly returned to basal levels within ten minutes when the attack subsided.

DR. B. T. HORTON, Rochester, Minn.—In 1939, at the meeting of this Society, Dr. H. L. Alexander gave me the idea of giving histamine intravenously, as a therapeutic agent. Since that time we have given 25,821 such injections, 13,400 of which have been given since I discussed the subject before this Society a year ago.

It was because of my interest in histamine that Doctor Roth asked me to see Patient 1, with paroxysmal hypertension, after she had failed to precipitate attacks by means of the cold pressor test of Hines as well as by the use of insulin and adrenalin.

I attempted to give the patient histamine intravenously by the drip method, using a 1:250,000 dilution according to our standard method. Histamine promptly precipitated an attack. The blood pressure, which Doctor Roth recorded, rose to a high level, as she has indicated. After a lapse of time I gave the same patient 0.05 mg. of histamine intravenously, and an attack developed within forty seconds, with a resulting rise in blood pressure from 130/110 to 280/295. I made a technicolored movie of the attack, which a number of the men in this audience have seen. Following surgical removal of the pheochromocytoma, histamine no longer precipitated attacks.

Why does a paradoxical rise in blood pressure occur following the injection of histamine intravenously? Histamine and adrenalin are physiologic antagonists. Schenk, in 1921, was apparently the first to call attention to this fact. A . . . . . when given intravenously in such a case causes a . . . . . into the blood stream. A pulsating type of head- . . . . . to the sudden rise in blood pressure. This is the basis of the syndrome of paroxysmal hypertension. This headache need not be confused with histaminic cephalgia, because it is generalized, whereas histaminic cephalgia is always unilateral.

Histamine can be used intravenously to bring out localizing signs and symptoms of other obscure tumors. I would like to refer briefly to the case of a woman aged 31 years with symptoms of two and a half years' duration, suggesting multiple sclerosis. During the course of twenty-two intravenous injections of histamine her symptoms became markedly worse. Signs of a cord lesion at the level of the tenth dorsal vertebra developed, and surgical exploration Oct. 17, 1944, revealed an intramedullary tumor 2 by 1½ cm. which was removed. She is now convalescing.

How did histamine act in this case? It caused a rise in intraspinal pressure, thus exaggerating the pressure symptoms of the tumor to the extent that the obscure lesion could be diagnosed clinically.

DR. H. NECHELES, Chicago, Ill.—We should be interested in the mechanism through which this effect is produced in cases of pheochromocytoma, and I wonder whether Doctor Roth has any suggestions. It may be a carotid sinus effect, and the same reaction may be obtained with other procedures affecting the carotid sinus.

Doctor Roth mentioned an amount of 50 to 1,000 mg. of adrenalin in the tumor. Is that in actual milligrams?

DR. ROTH (closing).—There has been some experimental work done by Kellaway and Cowell who injected histamine intravenously into a nonetherized cat and observed transitory slowing of the heart, dilatation of the pupils, and sweating. In an animal with intact adrenals, an injection of histamine produced dilatation of the denervated pupil. In an animal from which the entire adrenals or the medulla alone had been removed, the histamine caused no such dilatation. This was considered proof that histamine accelerated a secretion from the medulla of the adrenal glands.

In regard to the carotid sinus, Hyman and Mencher found that massage of the carotid sinus during the maximum rise in blood pressure caused a prompt decrease in the blood pressure which immediately rose when massage was stopped.

It was in actual milligrams.

## ISOIMMUNITY TO THE RH FACTOR AS A CAUSE OF BLOOD TRANSFUSION REACTIONS

ELMER L. DEGOWIN, M.D.

IOWA CITY, IOWA

Ever since the demonstration of isoimmunity to the Rh factor, incurred during pregnancy or after multiple blood transfusions, it has been difficult to obtain a proper perspective on the role of this phenomenon in the production of transfusion reactions in a general hospital service. During a period of eighteen months the blood transfusions in the State University of Iowa Hospitals were studied with respect to this problem. Suitable tests for anti-Rh agglutinins were made on the blood of all recipients exhibiting chills, fever, hemoglobinuria, or jaundice after transfusion. In 5,386 consecutive blood transfusions there were 186 reactions of all types, an incidence of 3.4 per cent. Of these, six were due to isoimmunity to the Rh factor, an incidence of 0.1 per cent in all transfusions, many of which were given to the same recipient or to pregnant women. In four cases isoimmunity was attributable to multiple transfusions and in two, pregnancies were responsible. One fatality occurred.

CASE 1.—A male physician, 37 years of age, after a gastric resection had three transfusions in one month without reaction. Two months later another transfusion produced chills, fever, and bilirubinemia. His blood proved to be Rh negative but no anti-Rh agglutinin could be demonstrated.

CASE 2.—A man 21 years of age had tuberculosis of the hip. He received ten blood transfusions in a month, the last few of which were accompanied by chills and fever. His blood was found to be Rh negative and contained an anti-Rh agglutinin. Two subsequent transfusions with Rh-negative blood were given without reaction.

CASE 3.—A woman aged 37 years received three blood transfusions without reaction in one week for an incomplete abortion. A fourth transfusion

after an interval of twenty-one days produced intravascular hemolysis. Her blood was found to be Rh negative and contained an anti-Rh agglutinin. Subsequent transfusion with Rh-negative blood produced no reaction. She had eight living children whose bloods were typed for the Rh factor. There had been no manifestations of erythroblastosis in any of her pregnancies.

CASE 4.—A woman aged 49 years was treated for metrorrhagia with a blood transfusion which was followed by chills and fever. Another transfusion was given the next day which resulted in hemoglobinuria and jaundice. Her blood was found to be Rh negative and contained an anti-Rh agglutinin of extremely high titer. Three subsequent transfusions with Rh-negative blood gave no reactions. Seven pregnancies had occurred more than ten years before, resulting in three living children, three abortions, one stillbirth, and one infant dying from umbilical hemorrhage.

CASE 5.—A woman aged 25 years was given a blood transfusion for toxemia of pregnancy. Chills, fever, dyspnea, bilirubinemia, and bilirubinuria followed, but at no time was there free hemoglobin in the blood stream. A dead fetus was promptly delivered which had the typical stigmas of erythroblastosis. The patient's blood was Rh negative and that of the baby was Rh positive, but no anti-Rh agglutinin could be demonstrated in the mother's blood. Two weeks later such an agglutinin had appeared.

CASE 6.—A woman aged 30 years had never been pregnant. Eleven years previously she had received three blood transfusions and a year later, two more, in the course of a lobectomy and the drainage of a subphrenic abscess. There had been no reactions. In July, 1944, another transfusion was given without reaction. Twelve days later another blood transfusion was followed by chills, fever, and hemoglobinuria. Oliguria ensued and, although decapsulation of the kidneys was performed on the third day, she died on the twelfth day in uremia. Her blood was found to be Rh negative and contained an anti-Rh agglutinin which reacted with the Rh-positive blood which she had received.

*Conclusions.*—1. Isoimmunity to the Rh factor was detected in 0.1 per cent of 5,375 consecutive blood transfusions and accounted for only 2.4 per cent of the reactions of all types which were noted in the series.

2. Routine compatibility tests for the detection of an anti-Rh agglutinin would have prevented four out of the six reactions reported in this series. They would have prevented one fatality in over 5,000 blood transfusions. These tests will not prevent all reactions due to isoimmunity to the Rh factor.

3. It is recommended that suitable compatibility tests for the detection of the Rh factor be performed before transfusion of any recipient who has ever been pregnant or has received transfusions more than one week previously.

#### DISCUSSION

DR. C. C. STURGIS, Ann Arbor, Mich.—I should like to ask Doctor DeGowin what he means by "routine compatibility tests" and also have him amplify his statement that tests for Rh agglutination would have prevented four out of the six reactions. I assume he wished to indicate that the blood of both the recipient and the donor should have been typed for the Rh factor and Rh-negative blood should then have been given to the Rh-negative recipient. May I also

ask him to express his opinion about the effectiveness of the compatibility tests with respect to the Rh factor in which the red blood cells of the donor are brought in contact with the recipient's serum at room temperature and when they are incubated at 37° C. for one hour.

DR. C. J. WATSON, Minneapolis, Minn.—If one carries out the hemolysis test for an hour in the incubator, would that be sufficient to exclude a reaction of this type?

DR. W. J. DIECKMANN, Chicago, Ill.—I wish, as an obstetrician, to endorse Doctor DeGowin's comments about the importance of the Rh blood group, especially, as I am a convert.

We recently had two Rh-negative patients, but without any demonstrable anti-Rh agglutinins, who had given birth to babies with erythroblastosis, and gave 100 c.c. of Rh-positive blood to one and 50 to another. Each patient had a severe reaction and increased bile pigments in the serum.

Within the past month an Rh-negative patient was given two Rh-positive blood transfusions because no Rh-negative blood was available. In such cases sodium bicarbonate should be given in 4 Gm. doses every two hours for twelve hours, or if the patient is in shock, 300 c.c. molar sodium lactate dissolved in 2,700 c.c. 5 per cent glucose should be given half intravenously and the remainder by clysis.

DR. DEGOWIN (closing).—With regard to Doctor Sturgis' question about compatibility tests, there is a distinct difference in the way antiserum containing Rh agglutinins reacts in the ordinary laboratory tests and the reactions of naturally occurring isoagglutinins. In performing cross-matching of blood with regard to the ABO system, no incubation is required. Any procedure which brings the agglutinins and agglutinogens in contact and then produces sedimentation is perfectly satisfactory. In looking for the anti-Rh agglutinins, it is apparently necessary to incubate the antiserum with the cells at 37° C., usually for a period varying from twenty to sixty minutes. The procedure also differs in the care which must be taken in manipulating cell suspensions to prevent excessive shaking. Ordinarily, with anti-A and anti-B sera, the resulting cell clumps cannot be shaken apart. On the other hand, clumps formed by anti-Rh sera are frequently small and are readily broken up by agitation so that the reaction may be entirely missed. The performance of tests involving anti-Rh agglutinins is difficult and requires much more experience than when natural agglutinins are concerned. Even as at present performed, agglutination may be easily overlooked.

With regard to Doctor Watson's question, searching for hemolysis in the test tube is inadequate to demonstrate incompatibility to the Rh factor. Although hemolysis occurs when incompatible blood is introduced into the body, no one has succeeded in demonstrating hemolysis in the test tube in this type of incompatibility.

Another clinical fact which has not been dealt with in this paper is that the tissues of the baby with erythroblastosis fetalis contain the anti-Rh antibodies of the mother. If the baby, whose blood is Rh positive, is transfused with more Rh-positive blood, the transfused cells are quickly hemolyzed. The child should receive Rh-negative blood.

## EXPERIMENTAL STUDY OF ANURIA IN BURNS

WILLIAM H. OLSON (BY INVITATION) AND H. NECHELES, M.D.  
CHICAGO, ILL.

We have reported previously that the anuria occurring in shock can be classified into three different types. The anuria following thermal trauma begins immediately after the burn. The present report deals with a comparison of various intravenous fluids used in order to overcome this acute anuria.

Animals were used under permanent anesthesia; all experiments were acute. The degree of hemoglobinemia was the most reliable index of the severity of the anuria.

Plasma and gelatin had very little therapeutic value in restoring urine secretion. The following solutions have some value: lactate plus 5 per cent glucose and 2 per cent urea; saline plus 2 per cent urea; Tyrode's solution; and saline with .87 per cent sodium bicarbonate. Isotonic sodium sulfate has proved to be superior to most fluids in overcoming the anuria due to burns.

## DISCUSSION

DR. NECHELES.—We believe that the free plasma hemoglobin following burns is an indication of the severity of the burn. In the experiments reported by Mr. Olson, we can be sure that the dogs with very high values for free plasma hemoglobin were the more severely burned, and we have grouped our experiments accordingly. It is interesting that in the reports on burns in human beings most authors do not report on free plasma hemoglobin. We know that hemoglobin is quickly taken care of by the reticulo-endothelial cells and by the kidneys. We feel that the concentration of free plasma hemoglobin is related to the degree of kidney damage. Experiments to determine this are under way. So far, sodium sulfate seems to be the best solution to overcome the anuria of burns in acute experiments.

THE DISTINCTION OF HEMOLYTIC AND NONHEMOLYTIC  
TRANSFUSION REACTIONS

EDMUND B. FLINK, M.D.  
MINNEAPOLIS, MINN.

(INTRODUCED BY W. W. SPINK, M.D., MINNEAPOLIS, MINN.)

Transfusions of whole blood or red blood cells may be attended by reactions. The majority of these are simple febrile or urticarial types, but some are hemolytic. The recognition of hemolytic transfusion reactions and their distinction from those of simple febrile type are often matters of considerable practical importance.

The present study of transfusion reactions has depended upon the quantitative determination of hemoglobin in blood plasma and urine. Artificial hemolysis was prevented by drawing the blood carefully into isotonic sodium citrate solution. The hemoglobin concentration of the plasma was determined by the method of Flink and Watson, in which hemoglobin is converted to pyridine hemochromogen and measured by means of an Evelyn photoelectric colorimeter. Normal values with this method range from a trace to 5 mg. per 100 c.c. of plasma. With hemoglobin concentrations of from 20 to 25 mg. per 100 c.c. the presence of hemolysis is evident to the eye.

## FACTORS CONTROLLING THE CORONARY CIRCULATION

LOUIS N. KATZ, M.D., WILFRED WISE, M.D. (BY INVITATION), AND  
KENNETH JOCHIM, PH.D. (BY INVITATION)  
CHICAGO, ILL.

For the past dozen years we have been investigating the control of the coronary circulation on preparations in which the coronary vessels were perfused from a separate circuit. In the intact animal, however, the coronary vessels are perfused from the same trunk, the aorta, which feeds the other systemic circuits. For this reason, we have recently analyzed seventy-nine experiments on the isolated denervated heart and heart-lung preparations in which this normal parallel circuit arrangement was permitted to exist. In these experiments, the coronary flow was measured by the difference in flow between the main pulmonary artery and the aorta simultaneously determined with modified Ludwig flowmeters.

The coronary flow was found to depend upon the cardiac output per minute, *often independently of the aortic pressure level*. This correlation between cardiac output and coronary flow of the denervated heart is explained on a purely mechanical basis. When the output of the left ventricle increases, blood is forced at an increased rate through both the coronary and the other systemic circuits. This increased flow tends to distend these circuits, thereby reducing their resistance, since resistance in a system of elastic tubes is an exponential function of vessel caliber. This automatic decrease in resistance may be sufficient in many instances to prevent any significant rise in aortic pressure.

In addition, with the cardiac output constant, changes in the peripheral resistance of the extracoronary systemic circuit caused parallel changes in coronary flow, sometimes without any significant alteration in the aortic pressure. This change in coronary flow is determined by changes in the resistance of the extracoronary paths per se. The total peripheral resistance may not change appreciably if the coronary resistance is, at the same time, sufficiently decreased by the passive distention caused by the diversion of blood to the coronary bed, so that aortic pressure may not change appreciably.

Thus, in the intact circulation coronary flow depends primarily on two passive factors: (1) the cardiac output per minute and (2) the state of constriction of the extracoronary systemic blood vessels. Furthermore, changes in these two factors can modify coronary flow independently of obvious arterial blood pressure changes.

These facts, of course, do not negate the value of powerful coronary vasodilator drugs to supplement such mechanical (and other possible humoral) regulators. However, in employing such drugs care should be taken (1) that they are powerful enough, (2) that they do not at the same time increase the work of the heart proportionately (or out of proportion), and (3) that they do not divert blood from the coronary circuit by an equal or greater dilating action upon the other systemic vascular beds.

## DISCUSSION

DR. JOHN R. SMITH, St. Louis, Mo.—I was rather cheered to hear Doctor Katz say that he was shocked at some of his results, because I ran into that in



some of my experiments. I started out to perfuse one of the auricular arteries and to study perfusion into the auricular arteries under ordinary tension. In noting the perfusion of blood into the left anterior auricular artery, I found that by raising the cardiac output without changing the blood pressure, inflow into this little artery increased three or four times. Fearing that something was wrong in my experiments, I repeated them three or four times. Apparently the same thing as Dr. Katz noted with the total coronary artery inflow was what I noticed with only one little twig of the coronary system.

DR. EMMET B. BAY, Chicago, Ill.—I would like to ask Doctor Katz what he thinks the mechanism is that makes the dilatation in the coronary arteries. Something has to dilate the coronaries and possibly that very slight increase in pressure would serve to dilate them. In the other experiment it is also quite clear, as Doctor Katz pointed out, that if carried to absurdity the vessel would have ruptured; this in Doctor Katz mind must mean quite an increase in pressure. I do not believe it is fair to talk in terms of constriction at the root of the aorta determining coronary flow without a change in pressure since other work has quite clearly demonstrated that by changing the pressure at the root of the aorta it is possible to change the coronary flow.

DR. MOSES BARRON, Minneapolis, Minn.—I would like to ask Doctor Katz if the coronary flow is increased in cases of coarctation of the aorta.

DR. KATZ (closing).—I am delighted to hear that Doctor Smith had a similar experience on perfusing a single coronary artery.

Doctor Bay's point is well taken. In my presentation, I was careful to state that there was no appreciable change in aortic pressure. We do not deny that the pressure rose, but the rise was often tiny, less than 1 mm. of mercury, despite the large augmentation of the coronary flow. Such a rise in blood pressure is of no importance clinically and would be considered as no change. Furthermore, the pressure change in the aorta is no criterion of the pressure change in the smaller coronary vessels. Even if the aortic pressure changes little, it is still possible that the pressure in the smaller vessels will rise appreciably. Distention of the coronary vessels will cause the pressure gradient to fall; therefore, if the aortic pressure does not rise significantly the pressure in the small coronary vessels should on this account rise definitely. Our argument is thus directed against the idea that aortic blood pressure change is the sole factor which alters coronary flow passively. We agree, and have ourselves demonstrated, that coronary flow increases when the aortic pressure goes up, but in the intact animal we insist that coronary flow may increase greatly with practically no change in aortic pressure.

As to Doctor Barron's question about coronary flow in coarctation of the aorta, I would be inclined from our experiments to believe that the coronary flow is definitely increased because the coarctation is equivalent to the screwing down of the screw clamp on the aorta of our heart-lung preparation. When the coarctation is small, there may be no rise in aortic pressure proximal to the lesion and yet the coronary flow will be increased. With more severe lesions the pressure proximal to the lesion will also go up.

## THE USE OF PAPAVERINE IN CORONARY ARTERY DISEASE

L. W. SWANSON, M.D.

MASON CITY, IOWA

Since the very favorable report by Katz on the efficacy of papaverine in large dosage in the treatment of angina pectoris, twelve patients with varying degrees of coronary insufficiency have been treated with papaverine. Their ages varied from 42 to 74 years. Four of the twelve had angina of effort but no evidence of coronary occlusion either by history or electrocardiographic examination, while the remaining eight all gave a history of healed coronary occlusions which had been confirmed by observation and/or typical changes in the electrocardiographic pattern. The duration of symptoms varied from two months to one year. All of the patients complained that their respective activity levels were much reduced by the anginal pains; three of them had actually been bedridden by the severity and frequency of the attacks. Papaverine hydrochloride was administered orally in doses of  $1\frac{1}{2}$  gr. four times daily.

Eleven of the twelve patients appeared to be definitely improved by the drug. One did not, and the administration of the drug had to be stopped because of excessive sleepiness. In none of the other cases was oversedation noted as a disturbing side-effect. No suggestion of addiction was observed upon cessation of the treatment. Two of the bedridden patients were improved enough to tolerate the activity of being up enough to care for themselves at home after approximately three and five weeks of treatment, respectively. Two of the others who had been forced by their attacks to quit all manual labor were able to resume light work after one and three weeks of papaverine administration. The remainder of the eleven patients who improved measured their improvement by stating they had fewer attacks of pain and could walk farther.

These results are thought to be significant even in a small series because of the high proportion who were improved and because all of the patients had previous therapeutic trials on conventional methods.

## DISCUSSION

DR. MOSES BARRON, Minneapolis, Minn.—I would like to ask the essayist how long he keeps up this treatment of  $1\frac{1}{2}$  gr. four times a day.

DR. SWANSON (closing).—One patient has been on this treatment almost continuously for fourteen months. Whenever he gets into trouble he wants to resume it.

## STUDIES ON THE CHANGES IN THE CIRCULATION OF NORMAL SUBJECTS WITH SMALL VARIATIONS IN THE FLUID CONTENT OF THE BODY

RICHARD H. LYONS, M.D., FRANKLIN D. JOHNSTON, M.D., AND  
JOHN SANDERS, B.S. (BY INVITATION)

ANN ARBOR, MICH.

The administration of 2 c.c. of mercupurine to ten normal subjects resulted in a mean loss of weight of  $1.76 \pm .3$  kg., a decrease in the plasma volume of  $544 \pm 88$  c.c., and a decrease in venous pressure of  $25 \pm 5$  mm. of water. The

significance of these changes was further studied in seven other normal subjects on whom twelve observations of the cardiac output, stroke output, and heart rate were made with the ballistocardiogram twenty-four hours after a 2 c.c. injection of mercupurine. These observations were compared to thirty-five control studies. There was a mean decrease in the stroke volume of 17 per cent; in the cardiac output per minute, of 12.4 per cent. in the venous pressure, of 15.8 per cent; and in body weight, of 1.9 per cent. The heart rate increased 9.5 per cent and there was an 8.2 per cent increase in the diastolic pressure. These changes were statistically significant when compared to the control observations. Some subjects complained of weakness, tiredness, and faintness on standing.

The oral administration of large amounts of sodium chloride (20 Gm.) or sodium bicarbonate (25 Gm.) is associated with an increase in plasma volume, 400 to 440 c.c., a rise in venous pressure of 17 to 30 mm. of water, and a gain in body weight of 1.6 to 1.9 kg. Eleven observations on seven normal subjects after the ingestion of 25 Gm. of sodium chloride per day for two days showed a mean increase in stroke output of 9.4 per cent. in cardiac output, of 4 per cent; in systolic blood pressure, of 7.2 per cent; in venous pressure, of 28.8 per cent; in body weight, of 2.2 per cent. There was a decrease in the heart rate of 5.3 per cent. Except for the increase in cardiac output and decrease in heart rate, these changes were statistically significant when compared to the control observations.

It is concluded that small changes in the extracellular fluid content of the body of 1.5 to 2 liters is sufficient to produce changes in the plasma volume, venous pressure, stroke output, and pulse pressure.

#### DISCUSSION

DR. G. T. EVANS, Minneapolis, Minn.—Has there been any observation made on increasing the intake of plain water? I am thinking particularly of the experiment of Margaria in which a larger intake of fluid was not accompanied by a dilution of the blood.

QUESTION.—May I ask if Doctor Lyons has any observations on the hematocrit, hemoglobin, or the plasma-protein of the blood.

DR. LYONS (closing).—In measuring plasma volume we have also done hematocrits and have found in general that percentage change in plasma volume determined by the dye method may be considerably more than the percentage of change of the hematocrit. The hematocrit has not been a good index of change in the plasma volume. The same is true of the serum protein concentration which does not change as much as the plasma volume determined by the dye method. This may be due to the change in total circulating protein. Roughly, when we have an increased plasma volume, we get an increase in the circulating protein, and with a decrease in plasma volume there is a decrease in total circulating protein; therefore the change in protein concentration will not vary as much as the change in plasma volume.

We have made no experiments on the viscosity of the blood so I cannot comment on that.

Regarding the change in plasma volume with the ingestion of large volumes of water, we have observations on medical students who have ingested 2 liters of water and we have found significant change in the plasma volume.

DEVELOPMENT OF A TEST FOR THE DETECTION OF ANTIBODIES  
IN SERUM OF TUBERCULOUS HUMAN BEINGS AND GUINEA PIGSR. O. MUETHER, M.D., AND WILLIAM C. MACDONALD, M.D. (BY INVITATION)  
ST. LOUIS, MO.

The serologic diagnosis of tuberculosis has always been unsatisfactory, but the desirability of having a satisfactory procedure has never been questioned.

Cannon and Marshall, using collodion pellets coated with old tuberculin, have devised a technique which has been useful in their hands. We have been interested in this problem for a number of years and have attempted to develop a satisfactory technique basing our work on that of Roberts and Jones with virus agglutination tests.

The technique used at present consists of coating a nonpathogenic organism, *Serratia marcescens*, with old tuberculin. To secure a satisfactory antigen, it has been found necessary to have a uniform suspension of organisms of constant density and it is important that the ratio of bacterial N to old tuberculin nitrogen be adjusted carefully.

Once a satisfactory antigen is prepared, the antibodies, if present in serum, will cause precipitation and the titer of the antibodies can be determined by making serial dilution of the serum.

In the present study, infected guinea pigs as well as human subjects were used.

The guinea pigs were infected with suspensions of tubercle bacilli and then bled at four-day intervals for twelve days. Each serum was tested for antibodies. The serum of the infected animals gave positive tests in increasing titer as the infection progressed, while the control animals remained normal.

Studies on human sera have shown negative reactions when applied to "tuberculin-negative" individuals, while "tuberculin-positive" patients without active tuberculosis showed a weak reaction (1:64). Active cases of pulmonary tuberculosis gave high titers up to 1:1024. Arrested cases showed much lower titers ranging from 1:32 to 1:256.

## TOXICITY OF SALICYLATES\*

MAJOR FREDERICK S. COOMBS, MAJOR HARRY A. WARREN (BY INVITATION),  
AND LIEUTENANT COLONEL CHARLES S. HIGLEY (BY INVITATION)  
MEDICAL CORPS, ARMY OF THE UNITED STATES

Recent papers have advocated the establishment of plasma salicylate levels of between 30 to 40 mg. per 100 c.c. without the use of sodium bicarbonate in the treatment of acute rheumatic fever.

We have studied a series of eighty-four patients with acute rheumatic fever in whom the plasma salicylate levels have been followed during intravenous and oral administration of from 10 to 16 Gm. daily. While it is too early to evaluate the efficacy of such treatment in preventing cardiac lesions, we have been impressed with the toxic reactions occurring in such patients when plasma salicylate levels are maintained at over 30 mg. per 100 c.c. Previous investigators

\*From Truax Field, Madison, Wis.

have reported partially on this syndrome, but before salicylate levels were available. Re-emphasis now is deemed important in view of current interest in salicylate therapy in acute rheumatic fever.

The toxic reactions, or salicylism, may be divided into three parts:

1. Respiratory alkalosis and its concomitant changes
2. Pustular acne
3. Maniacal delirium

The respiratory alkalosis is characterized clinically by an increase in the rate and depth of respiration, at times severe enough to cause numbness and tingling of the acral parts and on one occasion to produce carpo-pedal spasm. Chemically the blood shows a rise in pH, a lowering of  $\text{CO}_2$  content, rise in serum chlorides, early compensatory loss of sodium, and retention of water. Decreased renal function measured by urea clearance and intravenous phenol-sulfonephthalein tests results. Charts demonstrate these changes.

When sodium bicarbonate is administered along with sodium salicylate, lower blood levels are obtained, and the respiratory alkalosis does not develop principally because sodium bicarbonate increases the excretion of salicylates.

Colored slides illustrate the pustular acne which simulates bromidism. The effect on the central nervous system is outlined.

The theory that accumulation of salicylates in the blood leads to direct stimulation of the respiratory center and thus to hyperventilation alkalosis is propounded. Continued alkalosis of the degree observed in some of our patients is severe enough to interfere with kidney function and may lead to permanent renal damage.

#### DISCUSSION

DR. WALTER L. PALMER, Chicago, Ill.—The renal changes are not proof of renal injury. Doctor Kirsner, in his study of alkalosis, did not find definite evidence of renal damage. The changes in function return to normal when the electrolyte balance becomes normal. In other words, the alteration in renal excretion may be purely a reflection of the disturbed electrolyte balance.

DR. A. J. QUICK, Milwaukee, Wis.—I think that these new clinical studies employing large doses of salicylates should stimulate further investigation concerning the metabolism of salicylic acid. It is to be remembered that salicylic acid is really a phenol with the properties of the phenolic group modified by the carboxyl group. Salicylic acid is slowly eliminated from the body—even after taking only 1 Gm. of sodium salicylates, salicylic acid can be detected in the urine for twenty-four hours and often for forty-eight hours. We know that salicylic acid undergoes various metabolic changes in the body. Some of it is combined with glycine; some is oxidized to a dihydroxy salicylic acid. We do not know much about these polyhydroxy compounds, but it is probable that they may exert a toxic action on bone marrow and nerve tissue. Since salicylates are now used in massive doses, it might be well to investigate further the fate of salicylic acid in the organism. Coburn has done some of this work but more study is necessary.

DR. C. J. WATSON, Minneapolis, Minn.—Did you follow the prothrombin or fibrinogen levels in these patients?

MAJOR COOMBS (closing).—We, of course, were interested in knowing whether the prothrombin time was changed. We had some patients with a blood salicylate level of approximately 35 mg. and found the results were entirely normal. There was no effect on the prothrombin. We did not do any test for fibrinogen.

## SUBACUTE BACTERIAL ENDOCARDITIS CONFINED TO THE RIGHT SIDE OF THE HEART AND ENDARTERITIS OF THE PULMONARY ARTERY

PAUL S. BARKER, M.D.

ANN ARBOR, MICH.

The usual clinical features of subacute bacterial endocarditis confined to the right side of the heart, and of subacute bacterial endarteritis superimposed on patent ductus arteriosus, are well known. It is also well known that they are extremely variable, that many cases deviate from the typical picture, and that diagnosis is often difficult. Nevertheless, the success of surgery in curing patent ductus in which infection has not yet involved the heart valves makes exact diagnosis imperative. For aid in this problem, a study of the University Hospital cases and a review of the literature were undertaken.

The physical signs usually indicate clearly a lesion of the right side of the heart or patent ductus arteriosus. Sometimes the signs are atypical, the murmurs confusing or absent. Murmurs often change and occasionally disappear during the course of the disease, due probably to changes in the vegetations which may occlude a patent ductus or alter the direction of flow through a septal defect by obstructing the pulmonary artery. Systolic and, occasionally, diastolic murmurs may be heard in the mitral or aortic area in the absence of lesions of these valves. Conversely, these valves may be involved without producing characteristic signs. In endarteritis complicating patent ductus, involvement of the aortic and mitral valves is usually obvious, but involvement of the pulmonic valve is often obscured.

Signs of infection are present, such as fever and tachycardia. Anemia usually appears early. The leucocyte count varies but is usually moderately elevated. Abdominal discomfort, anorexia, nausea, wasting, and cough are common. Blood cultures are usually positive; sometimes they are sterile, especially early in the disease.

Infarction of the lungs is usually, but not always, present. Peripheral emboli in the greater circulation are rare and require special explanation. In some instances, the flow through a septal defect or a patent ductus may be altered so that it is from right to left. In other cases with no abnormal communication, one may speculate regarding the possibility of infected pulmonary infarcts causing pulmonary venous thrombosis which in turn gives rise to peripheral emboli. Enlargement of the spleen is common and is usually due to acute splenic tumor accompanying the infection or to passive congestion, occasionally to infarction. Albuminuria and microscopic hematuria are common, and autopsy usually reveals glomerulotubular nephritis; rarely renal infarction or focal embolic nephritis is discovered. Petechiae sometimes appear, occasionally purpura.

### DISCUSSION

DR. M. J. SHAPIRO, Minneapolis, Minn.—I would like to comment on the diagnosis of patent ductus without a typical murmur. One can conjecture as to whether he has a patent ductus if the murmur is not typical but I would suggest that such a diagnosis be not made preoperatively. If the murmur is not typical and surgery is done on many of these patients, a lot of mistakes will be made.

A patient with subacute infection superimposed on a patent ductus should be operated because he has nothing to lose. Even with penicillin and the sulfonamides we have nothing available. In a large percentage of cases of subacute bacterial endocarditis, cure has been obtained by surgery alone.

DR. C. J. WATSON, Minneapolis, Minn.—I should like to ask Doctor Barker whether he has encountered right-sided bacterial endocarditis in patients with pulmonary tuberculosis and whether he knows if tubercle bacilli may cause subacute bacterial endocarditis. We recently had a patient who had an extensive pulmonary tuberculosis with cavities. He presented a very loud systolic murmur in the pulmonic area but had no evidence otherwise of subacute bacterial endocarditis with the exception of an enlarged spleen. This patient was discharged to a tuberculosis sanitarium at some distance. We were told subsequently that soon after arriving there he developed a severe purpura and at autopsy he had vegetations on the pulmonary valve, but no bacteriologic studies were made. We did not presume that this was a tuberculous endocarditis but it raised the question in our minds and I would like to know what you think about it.

DR. BARKER (closing).—In one of our cases a clinical diagnosis of subacute bacterial endocarditis in an interventricular septal defect was made. The patient left the hospital against advice and did not continue with sulfonamide treatment. Later, he turned up as a patient with tuberculosis of the lungs which he did not have at the time he had the bacterial endocarditis. He had recovered from the bacterial endocarditis and had normal temperature and negative blood cultures. In the literature there are reported several patients with right-sided subacute bacterial endocarditis and tuberculosis of the lungs and one with tuberculosis of the kidneys. I did not encounter any with tubercle bacilli implanted on the valves or on the endocardium.

I agree that operation should be restricted to patients with typical murmurs. Peripheral embolism is relatively uncommon and should suggest involvement of the mitral or aortic valves. In our limited experience some patients have responded to penicillin therapy and apparently have recovered from their infection following its use.

#### A CORRELATION OF COMPOSITE LIVER FUNCTION STUDIES WITH HISTOLOGIC CHANGES IN THE LIVER AS NOTED IN BIOPSY MATERIAL

F. W. HOFFBAUER, M.D.

MINNEAPOLIS, MINN.

(INTRODUCED BY C. J. WATSON, M.D.)

Clinical studies of liver function in various forms of jaundice and liver disease indicate the relative futility of using but one or two procedures intended to measure liver function. In the present study a battery of liver function tests has been employed, and the results have been recorded graphically in the form of a liver function "profile" or composite record. The present material consists of forty-eight cases in which composite studies were made and in which a total of fifty-three needle biopsies of the liver were carried out. This includes twelve cases in which, because of relative inaccessibility of the liver, the biopsy was done at the time of laparoscopy. This is to say that a biopsy needle was inserted directly through the anterior abdominal wall; the insertion into the liver was guided by visualization through the peritoneoscope. The employment of a needle allows sampling of liver tissue to a greater depth than is per-

mitted with the peritoneoscopic biopsy forceps, thus eliminating the objection to the latter method that only subcapsular material is obtained. For the ordinary biopsy (thirty-six cases), in the presence of an enlarged and easily palpable liver, the procedure was carried out at the bedside using an 85 mm., 14-gauge Silverman needle, as first employed for this purpose by Tripoli and Fader. The biopsy under peritoneoscopic control was done with a modified 180 mm. Silverman needle of the same gauge.

The composite study of liver function has included the following procedures for the nonjaundiced patient: quantitative fractional serum bilirubin; hippuric acid (one hour, intravenous method); fractional serum proteins; cephalin-cholesterol flocculation, bromsulfalein 2 mg. per kilogram, with twenty-minute reading; urine urobilinogen in milligrams per twenty-four hours. In the jaundiced subject the same procedures, with exception of the bromsulfalein test, are used, together with the total serum cholesterol, the serum phosphatase, the Quick prothrombin time (before and after vitamin K), and the feces urobilinogen (milligrams per day). The renal function factor in the hippuric acid test is evaluated by means of a simultaneous one-hour phenol-sulfonephthalein excretion test (1 c.c. of the dye is injected together with the sodium benzoate). In instances where the results with these standard procedures were conflicting or doubtful, supplementary and more sensitive tests were carried out. These included the bromsulfalein 5 mg. per kilogram thirty- and forty-five minute reading and the bilirubin tolerance test in the nonjaundiced subjects; the intravenous galactose test, stercobilin tolerance test, and urine coproporphyrin in both jaundiced and nonjaundiced subjects.

It is believed that the conjunction of the composite liver function study with liver biopsy has permitted much additional insight into the pathologic physiology of jaundice and liver disease. From these studies it has become increasingly apparent that cirrhosis of similar anatomic extent may exhibit strikingly different degrees and types of liver functional impairment. The concept of a pericholangitic type of hepatitis with normal hepatocellular function has become increasingly clear as a result of these correlations. The diagnosis of obscure or uncommon liver disorders, as, for example, primary amyloidosis, has been established by biopsy in cases in which the liver function profile, alone, although abnormal, was not diagnostic.

The study indicates that if a large enough series of correlations of the above type become available, the resulting patterns may provide information both as to the anatomic type of hepatic disease, and to the prognosis.

#### DISCUSSION

LIEUTENANT COLONEL JOHNSON McGUIRE, Cincinnati, Ohio.—I would like to ask if there has been any mortality in securing these biopsies.

DR. C. J. WATSON, Minneapolis, Minn.—I might add a word about liver biopsy, having watched Doctor Hoffbauer's procedure repeatedly. He has done somewhat more than sixty-five without untoward incident. An accident did occur in one case where the biopsy was done by a visiting physician, although under supervision. In this instance the needle undoubtedly went through the thin left lobe of the liver and damaged a vessel behind it. An alarming hemorrhage resulted which, however, stopped spontaneously. The patient recovered completely. If one limits the procedure to cases where the liver is easily pal-



pable below the right costal margin, and obviously just beneath the abdominal wall, the biopsy is believed to be quite safe. In other cases needle biopsy can be carried out satisfactorily under peritoneoscopic control. When observed through the peritoneoscope, the amount of bleeding is small, a few drops welling up on the surface after removal of the needle, promptly followed by clotting and cessation of bleeding. The insertion of the needle is relatively painless if the parietal peritoneum is first carefully anaesthetized.

Dr. HOFFBAUER (closing).—Needle biopsy of the liver has not been attempted in any patient where there was a suspicion of intra-abdominal or intra-hepatic suppuration. The presence of a hemorrhagic tendency has also been regarded as a contraindication. No fatalities have occurred as a result of this procedure.

Biopsy of the liver, as carried out by the surgeon at the time of operation or by the endoscopist with the peritoneoscope forceps, has been criticized on the basis that if the material is secured from just beneath the capsule it may not reflect the actual structure of the liver elsewhere. This can be avoided by the operator if an attempt is made to remove tissue from a sufficient depth into the liver. The Silverman biopsy needle permits penetration to a depth of several centimeters and is therefore advantageous

### THE CEPHALIN-CHOLESTEROL FLOCCULATION TEST

LEO J. WADE, M.D., AND ELLEN EHRENFEST RICHMAN, M.S.

ST. LOUIS, Mo.

(INTRODUCED BY CARL V. MOORE, M.D.)

The present communication is an attempt to evaluate the cephalin flocculation test under a variety of circumstances. From a series of 1,500 tests, 500 were done on the sera of patients whose diagnoses were established beyond any reasonable doubt. The results may be summarized as follows:

	NEG.	POS.
I. Diffuse parenchymatous disease of the liver.....	2.8	97.2
II. Discrete lesions of the liver.....	46.7	53.3
III. Suspected lesions or dysfunction of liver.....	62.8	37.2
IV. No known lesion or dysfunction of liver.....	81.9	18.1

Negative reactions did occur in the presence of diffuse liver disease but the lesions were invariably minimal. Flocculation was not encountered in normal healthy controls, but in the presence of infection, allergic disease, and during the puerperium or neonatal period positive reactions were encountered which are thought to be "false positives." In twelve of thirteen instances studied, the sera of mother and newborn infant ran a parallel course suggesting that the causative agent passes the placental barrier. These facts are not incompatible with Hanger's hypothesis that the flocculation is due to an abnormal globulin constituent, although no correlation was possible with the albumin-globulin ratios. It is suggested that as in the case of the flocculation tests for syphilis, experience will make apparent those situations in which the test is not reliable.

The test is of greatest value in acute hepatitis, catarrhal jaundice, and cirrhosis or in the presence of any other diffuse parenchymatous liver disease. In these situations the test has prognostic as well as diagnostic value. The flocculation test is of no value in the differentiation of obstructive and nonobstructive jaundice. Other tests, particularly the bromsulfalein test is more useful in detecting focal hepatic disease.

## BILIVERDIN ICTERUS

MAJOR E. A. LARSON, MEDICAL CORPS, ARMY OF THE UNITED STATES, AND G. T. EVANS, M.D. (BY INVITATION), MINNEAPOLIS, MINN.

A direct colorimetric method for the determination of serum biliverdin is described; it employs the Evelyn colorimeter and depends for its validity on the increasing light absorption of biliverdin in passing from green to red wave lengths, as compared with the decreasing or unchanging absorption of bilirubin, reduced hemoglobin, and oxyhemoglobin in passing through the same range. Methemoglobin must be specifically excluded.

Normal sera contain no detectable biliverdin (less than 0.05 mg. per cent). Ninety-three observations were made on the sera of sixty-three consecutive patients with jaundice. The biliverdin values ranged from zero to 2.2 mg. per cent.

When biliverdin is present, the general trend is for the higher values to occur in patients with the higher values for total bilirubin; thus, the six highest values for biliverdin (over 1 mg. per cent) were in patients (three carcinomatous obstruction; two, common duct stone; one, catarrhal jaundice) having total bilirubin above 17 mg. per cent (average, 24.4 mg. per cent).

A significant level of biliverdine was found in every patient with the following: (a) carcinomatous obstruction of the common bile duct (twenty-six); (b) catarrhal jaundice (four); (c) cirrhosis with jaundice (five); (d) common bile duct stricture (five). Among the patients mentioned in whom biliverdin was uniformly present there were fourteen with total bilirubin under 10 mg. per cent (average, 5.9 mg. per cent); compared with this it is worthy of note that of the ten patients with common bile duct stone, five with an average total bilirubin of 7.1 mg. per cent had no biliverdin in their sera.

The remaining thirteen patients (six transfusion reactions; three, hemolytic anemia; one, leucemia; one, Hodgkins' disease; one, Banti's syndrome; one, cardiac decompensation) with average total bilirubin of 7.6 mg. per cent (range 3 to 30 mg. per cent) all had zero biliverdin.

The green tint of the patient was noted in those having as little as 0.3 mg. per cent biliverdin (total bilirubin, 9 to 20 mg. per cent).

Serial observations in fourteen patients showed that biliverdin can change markedly in from one to five days without respect to the direction of change of the total jaundice. In three of these patients, during a period when there was a notably high caloric intake, the biliverdin was observed to disappear despite absence of marked change in the total jaundice.

In general it may be said that biliverdinemia is a further evidence of regurgitation jaundice and has not been observed in hemolytic (retention) jaundice. The absence of biliverdinemia speaks against the diagnosis of jaundice due to cancer.

## PRIMARY SPLENIC PAN-HEMATOPENIA

CHARLES A. DOAN, M.D.

COLUMBUS, OHIO

The physiologic role of the spleen as a reservoir and regulator of the circulating cellular elements in the blood is well established. The selective pathologic sequestration and destruction of red blood cells (congenital hemolytic icterus), platelets (thrombocytopenic purpura), and granulocytes (primary splenic neutropenia), respectively, associated with an hyperplasia of highly phagocytic R-E cells, with or without demonstrable splenic enlargement, may each be treated successfully by splenectomy. Secondary, usually asymptomatic, decreases in one or more of the cell types not primarily attacked by the splenic phagocytes have been noted and reported in our previous communications dealing with each syndrome as a separate entity. If this erratic pathologic physiology of the spleen is the principle underlying etiology in the conditions described, it should not be surprising to encounter certain individuals in whom this organ failed entirely to discriminate between the blood elements passing through its confines, with a resulting peripheral hematologic and clinical picture superficially resembling the pan-marrow hypoplasia in aplastic anemia. It is the purpose of this report to define and differentiate such a newly recognized syndrome, to suggest a diagnostic designation descriptive of the fundamental underlying mechanism, to present clinical examples, and to recommend splenectomy as an effective therapy.

"Primary splenic pan-hematopenia" occurs both as a chronic relapsing, and as a relatively acute, clinical syndrome, either associated with or independent of the congenital or acquired splenic dyscrasias previously described. The adrenalin test will reveal an abnormal excessive sequestration of all normal circulating elements by the spleen through a manyfold temporary increase above the base line in the peripheral circulation. Sternal bone marrow aspiration will show pan-marrow hyperplasia of all normal elements without pathologic maturation arrest or significant qualitative abnormalities, or foreign cell invasion, a finding in sharp contrast with the acellular marrow in hypoplastic anemia, or the hyperplasia in metastatic myelophthisia.

At least five more or less clear-cut examples of this syndrome have been encountered in our series of 170 splenectomies for splenic dyscrasias. The first case is that of a 14-year-old girl, apparently normal at birth, but from 6 months of age until seen in our Clinic in October, 1943, she had suffered more or less constantly from profound nonicteric anemia, leucopenia with recurring superficial infections and nonspecific febrile episodes, and easy bruising. A diagnosis of hypoplastic anemia was made when all hematogenic therapy failed, but no blood transfusions were ever given and complete aplasia obviously never occurred. The first available blood study was made at 29 months of age: W.B.C. 4200, R.B.C. 1,210,000; Hb. 4.5 Gm., PMN 29 per cent, PME 1 per cent, lymphocytes 65 per cent, monocytes 1 per cent. On admission Oct. 13, 1943: W.B.C. 1600, R.B.C. 1,030,000, Hb. 3.5 Gm., reticulocytes 3 per cent, platelets 228,000 (normal 750,000), hematocrit 10, MCV 97 c.mm., MCH 34, MCHC 35 per cent; corrected sedimentation index 0.2 mm. (normal); erythrocyte fragility range 412-341 (normal); icterus index 7; van den Bergh normal; supravital differ-

ential, PMN 36 per cent, PME 18 per cent, lymphocytes 38 per cent, monocytes 8 per cent. Chest x-ray, electrocardiogram and liver and kidney function tests showed no abnormalities; basal metabolic rate +15. *Adrenalin test*: W.B.C. 1,450 to 10,250; R.B.C. 1,300,000 to 1,700,000; Hb. 5.3 to 6.5 Gm., platelets 313,000 to 911,000. *Sternal bone marrow*: hyperplastic grossly; myeloid: erythroid ratio, 4:1; supravital differential; neutrophilic myelocytes "C" 67 per cent, eosinophilic myelocytes "C" 20 per cent, lymphocytes 5 per cent, clasmatoocytes 2 per cent, megakaryocytes 3 per cent; normoblasts 30 per cent, late erythroblasts 20 per cent, early erythroblasts 22 per cent, megaloblasts 28 per cent, no foreign or atypical cell types seen. *Splenectomy*, Oct. 21, 1943, with 1 c.c. adrenalin injected directly into the splenic artery prior to ligation of splenic pedicle: preoperatively, W.B.C. 2100, PMN 798; postoperatively W.B.C. 17,000, PMN 7,140; R.B.C. 1,100,000 to 2,010,000; Hb. 4.3 to 7.8 Gm.; platelets 255,000 to 1,157,000. Spleen weight 475 Gm.; no perisplenitis or adhesions, no infarction; supravital studies, hypersequestration of PMN, PME, R.B.C., and platelets with increased number and activity of phagocytic clasmatoocytes; no hematopoiesis. By fourteen days postoperatively: W.B.C. 9,000, PMN 3,780; R.B.C. 3,050,000, Hb. 9.5 Gm.; platelets 4,740,000/c.mm. Clinical improvement was dramatic and has continued to the present time. First normal menses, Jan. 30, 1944.

Mrs. D. K., aged 24 years, was admitted as a medical emergency to the Hematologic Service, University Hospital, with vague symptoms for six months, subacute for four weeks, and acute for seventy-two hours. Increasingly profound prostration with tachycardia, palpitation, dyspnea, precordial pain, marked pallor with questionable fluctuating icterus during the last ten days, and nausea and vomiting for seventy-two hours, were the presenting complaints. The spleen extended to the umbilicus. F. H. negative. Admission hematologic data: W.B.C. 2,100, PMN 420, myelocytes "C" 210; R.B.C. 820,000, Hb. 2.8 Gm., platelets 16,000 reticulocytes 69 per cent, icterus index 100, van den Bergh indirect, 3.69; urobilinogen, strongly positive. *Sternal bone marrow*: grossly hyperplastic with microscopic pan-hyperplasia for all normal elements. Splenectomy was successfully accomplished five hours after admission; preoperative and postoperative data, without blood transfusion, were as follows: W.B.C. 2,100 to 4,950; R.B.C. 1,010,000 to 1,990,000, Hb. 2.8 to 5.6 Gm., platelets 16,000 to 114,000. Hematologic and clinical improvement continued in parallel without complications, the patient being discharged on the twelfth postoperative day. A summary chart of the five cases shows similar cellular response following splenectomy.

#### DISCUSSION

DR. HOWARD L. ALT, Chicago, Ill.—These observations of Doctor Doan are of unusual interest. I saw a patient last July, a woman aged 62 years, with a similar condition. The red blood count was 1.56 million; white blood count, 2,900, and platelets, 55,000. The icterus index was 12 units and the reticulocyte count, 4.8 per cent. The bone marrow was normally cellular, the myeloid:erythroid ratio was 4:1, and there was some shift toward maturity in the normoblastic cells.

Following splenectomy early in August there was gradual improvement in the blood picture and all the elements returned to normal levels in a month. Because of the nature of the bone marrow pattern and the slow recovery, it was

thought that the hematopenia was due to splenic inhibition of bone marrow function.

In going over some of our cases of pancytopenia seen in the past, there are several that could be classified as primary splenic panhematopenia. They were originally diagnosed as primary refractory anemia with hypercellular marrows. Splenectomy deserves more extensive trial in this group of patients.

DR. FRANK H. BETHELL, Ann Arbor, Mich.—I should like to ask Doctor Doan how he interprets the results of the adrenalin test in determining the indications for splenectomy and if he performs puncture biopsies of the spleen on his patients before advising splenectomy.

DR. R. W. HIXLE, Cleveland, Ohio.—We have had two cases similar to Doctor Doan's which have been relieved by splenectomy so far as the neutrophile count is concerned. In one patient a pre-existing anemia was improved after splenectomy and in one it was not. I wonder how Doctor Doan explains the absence of evidence of hemolysis? It was absent in our patients and in all of Doctor Doan's patients except the last one reported this morning.

If the sole pathologic physiology is sequestration in the spleen, I wonder why there is sometimes considerable delay in the return of the neutrophiles after splenectomy? This was true, particularly, in our two patients; in one the neutrophiles increased slowly over a period of one year. It would seem to me that if sequestration were the only factor, the neutrophiles should increase promptly after splenectomy. Also, in view of the well-known abnormal sphericity of the erythrocytes in familial hemolytic anemia, I wonder whether splenic sequestration can be considered to be the principal disturbance in this disease. Similarly, it is not possible to explain all the features of idiopathic thrombocytopenic purpura on this basis.

DR. C. J. WATSON, Minneapolis, Minn.—I think it might be emphasized that hemolysis does occur in excess in an appreciable number of cases of what is designated as refractory or aplastic anemia. If one studies hemoglobin metabolism in patients with this diagnosis it will often be found that there is an increase of red cell destruction. In our experience this has commonly been cyclic in character, sometimes in excess of a two- to fourfold magnitude and then again in a fairly normal range. The reticulocytes are not increased as they are in the characteristic cases of hemolytic jaundice. On the other hand, they are often not decreased as one might expect them to be with bone marrow hypoplasia. Counts of 1 to 3 per cent are not uncommon. We have had a few such patients splenectomized but we have not seen any enduring benefit following splenectomy. We have a record of one patient with a remission of about four months. The blood picture did not return to normal but improved so markedly that transfusions were unnecessary. Then the anemia recurred and the patient went on to exitus. We have evidently not observed the type of case to have had the good results Doctor Doan has noted.

DR. DOAN (closing).—In answer to Doctor Bethell, the adrenalin test, we feel, will usually reveal quite accurately the type and degree of cell sequestration which is currently occurring in the splenic parenchyma. This is approximately proportional to the size of the spleen. Furthermore, at the operating table it has been shown that the total number of circulating cells in the splenic artery is greater than the number found in the splenic veins, confirming the reservoir function of this organ. The histologically verified presence of increased numbers of highly phagocytic clasmatoocytes, containing recognizable blood cell elements corresponding to the specific cellular deficiencies which exist in the peripheral blood, give good reason to suspect this mechanism of abnormal cell destruction. When the bone marrow is found to be hyperplastic for the identical cell types, without evidence of delayed or abnormal maturation, an unusual

demand for these elements may be assumed. Finally, when successful splenectomy is followed by an immediate and sustained increase in all circulating blood cell elements, the primary pathologic role of the spleen in such syndromes would seem to be firmly established.

We prefer not to risk intraperitoneal hemorrhage from direct needle aspiration of the splenic parenchyma through the intact abdominal wall unless the adrenalin test and sternal marrow aspiration together fail to reveal the desired information.

With reference to Doctor Heinle's remarks, I did not mean to infer that splenic sequestration and clasmotocytic destructive phagocytosis were the sole mechanisms which led to circulating blood cell deficits. There are, of course, other factors which must always be evaluated. Reticulocytosis, or an increased icterus index with positive indirect van den Bergh, are not necessary as criteria of excessive red cell destruction or hemolysis. Doctor Watson emphasized this in his discussion. The hepatic and renal thresholds for pigment disposition are extremely important in this regard. As has repeatedly been emphasized, the integrity of the bone marrow must be known in each individual case. When the spleen is the sole primary pathogen, complete and permanent recovery follows splenectomy; when secondarily contributory only, partial re-equilibration only may be expected.

### THE ANEMIA IN EIGHT ADULT PATIENTS WITH SCURVY

RICHARD W. VILTER, M.D., AND ROBERT M. WOOLFORD, M.D. (BY INVITATION)  
CINCINNATI, OHIO

In the Cincinnati General Hospital recently we have observed ten male adults with scurvy, eight of whom had anemia. Hemolysis appeared to be one causative factor because of the accompanying reticulocytosis and moderate hemolytic jaundice. There was no evidence of iron deficiency or of external bleeding. In each case the anemia and the other lesions of scurvy responded to the administration of vitamin C. These observations are reported because the existence of a specific anemia of scurvy has been questioned.

Red blood cell counts ranged from 1.74 to 3 million and hemoglobin levels from 5.8 to 10.5 Gm. The cells were normocytic normochromic or moderately macrocytic hyperchromic. The white count was under 6,000 in five patients. Differential white counts were normal. Platelets occurred in normal numbers. Initial reticulocyte levels ranged from 3 to 10 per cent except in two patients in whom initial counts of 1 per cent were obtained.

Bone marrow obtained by sternal aspiration appeared hypocellular in some patients, normally cellular or slightly hypercellular in others. There was a relative increase in the number of red cell progenitors, the majority of which were at the normoblast and late erythroblast maturation levels. The granulocyte series was cytologically normal.

Icteric indices ranged from 10 to 22; the urine contained excess urobilinogen but no bile; and the blood serum gave the indirect van den Bergh reaction in each instance. The extent of ecchymoses, however, could not be correlated with the degree of jaundice, nor with the severity of anemia, and no increase in ecchymoses occurred after the patients were hospitalized.

In the hospital these patients were fed a diet free of vitamin C and low in the vitamin B complex. Specific medication was withheld for from two to

fourteen days until mental torpor and impending shock made the administration of vitamin C imperative.

Before vitamin C administration was begun, erythrocyte counts and hemoglobin levels remained unchanged in six patients and decreased in two although the reticulocyte counts remained elevated or rose slowly.

After daily administration of 500 mg of vitamin C, two patients showed a rise in reticulocytes from 5 to 10 per cent and from 2 to 19 per cent, the peak value, on the sixth and tenth days, respectively. In the other six patients, initial high reticulocyte levels were maintained for six to ten days. Red blood cell and hemoglobin increases began one week after the initial dose of vitamin C. Within three weeks, the levels reached 3.5 million and 12.5 Gm or more in all patients. Icteric indices became normal when the red cells began to rise. Repeated bone marrow aspirations on four patients showed reversion to normal.

#### DISCUSSION

DR. C. J. WATSON, Minneapolis, Minn.—These are extremely interesting and instructive cases but I must object to the view that they are necessarily hemolytic. I agree fully that the findings suggest hemolytic anemia. Urobilinogenuria, however, does not suggest hemolysis. There are many clear-cut cases of hemolytic anemia without urobilinogenuria, and, conversely, there are many with urobilinogenuria without increased hemolysis. The idea that urobilinogenuria is pathognomonic of hemolytic anemia should be abandoned. The findings suggest that there was considerable hepatocellular dysfunction. In addition to that, there may have been hemolysis. I agree that the increased reticulocytes are suggestive of the latter.

DR. JOHN L. STIFEL, Toledo, Ohio.—Could salicylates have been an etiologic factor in any of these patients? I have in mind a patient who twice developed anemia similar to this after having taken a course of salicylates for rheumatoid arthritis. The second time she developed this in spite of large doses of ascorbic acid.

DR. ARMAND J. QUICK, Milwaukee, Wis.—I would like to ask whether the bleeding time test was done, also a tourniquet test, and, if positive, how soon the latter responded after vitamin C therapy.

QUESTION: I would like to ask if a fragility test was done.

DR. STANLEY E. DORST, Cincinnati, Ohio.—Doctor Vilter, earlier in his discussions, indicated that previous investigators studying C deficiencies alone did not encounter the symptoms he describes. I wonder whether or not he was able to determine any reasons for the discrepancies between their results and his conclusions.

DR. VILTER (closing).—I expected to have Doctor Watson question the data favoring hemolysis. Since June we have been waiting for another patient with scurvy so that we might obtain stool urobilinogen levels. Unfortunately such a patient has not appeared.

Liver function tests were done because we wondered whether hepatic dysfunction was responsible for the urobilinogenuria. The prothrombin times, bromsulfalein excretion, and cephalin flocculation tests were essentially normal. The moderately low serum albumin levels could easily be explained by dietary protein deficiency.

There was no specific history of salicylate ingestion, and I do not believe salicylism played any part in the purpura or anemia.

We did not carry out bleeding and clotting times routinely. Those that were done were normal. Tourniquet tests were abnormal, but we did not repeat them during treatment. The tourniquet test is not sufficiently specific for use in the study of scorbutic patients.

I have wondered many times why our results and those of Mettier, Minot, and Townsend differ from Loesner's and others who believe that vitamin C plays no part in hematopoiesis. It is possible that those patients who had hematologic remissions before vitamin C was administered differed from our patients in the degree of vitamin C depletion. All our patients who were put to bed on a diet very low in vitamin C invariably became much worse. Loesner apparently had no such difficulty with much longer control periods than ours. Possibly bed rest alone led to improvement. Similar remissions have been observed frequently in patients with pellagra when put to bed on a B complex-deficient diet.

## THE INFLUENCE OF DICUMAROL ON PLATELET ADHESIVENESS

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In 1941 H. P. Wright presented a method for measuring the adhesiveness of platelets, that is, their tendency to adhere to each other or to surfaces with which they come in contact. She reported that a number of *in vitro* anticoagulants would cause a decreased adhesiveness and that the decrease was proportional to the amount of anticoagulant used.

The method consisted of rotating blood in glass tubes and making platelet counts at various intervals of time. The tube was constructed in such a way that the sides, which were formed by cover slips held in place with petroleum jelly, could be removed and stained. The rate of fall of the platelet count was taken as a measure of adhesiveness; the less rapid the fall, the less sticky were the platelets.

In this study an attempt was made to determine whether dicumarol, an *in vivo* anticoagulant having no *in vitro* action, would cause a similar decreased adhesiveness.

Dicumarol was given to each of thirty-three patients for a period of six days. The dosage was gauged by means of prothrombin time determinations in which Pohle and Stewart's modification of the Quick method was used. Usually, four determinations of platelet adhesiveness were made on each patient, one before the administration of the drug, two during, and one afterward, when the effect was beginning to decline.

It was found that dicumarol produced a definite decreased adhesiveness as measured by the method of Wright. In general, the greatest decrease in platelet adhesiveness corresponded to the greatest prolongation of prothrombin time. The drug produced no change in platelet count *per se*, an observation previously reported. The data presented constitute additional evidence in favor of dicumarol as a prophylactic agent of thrombosis.

## DISCUSSION

DR. RICHARD K. RICHARDS, Chicago, Ill.—I would like to ask Doctor Meyer if he believes there is any relation between platelet adhesiveness and blood viscosity and, second, how heparin affects the adhesiveness with the same circumstances and the same technique.



DR. MEYER (closing).—Helen Wright noted that heparin decreased the adhesiveness of the platelets; proportional to the dose of heparin, it decreased the adhesiveness but did not lead to complete disappearance of this function.

I cannot answer the question regarding blood viscosity

### BLOOD AND BONE MARROW FINDINGS IN INFECTIOUS MONONUCLEOSIS

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LIEUTENANT COLONEL ROBERT M. JONES, MEDICAL CORPS, ARMY OF  
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Detailed studies of the blood and bone marrow were performed on a group of twenty-five patients with infectious mononucleosis during various stages of the disease. Eighteen men and seven women comprised the group. Fourteen ranged in age from 16 years to 26 years, four were less than 16 years of age, and one was over 30 years of age. No Negroes were observed in this series.

The leucocyte count varied between 4,000 to 22,000. In ten patients the count was less than 10,000 and two of these had a white count below 5,000. Only one patient had a count of over 20,000. A normocytic anemia was seen in three patients and a severe microcytic and hypochromic anemia was observed in another. The platelets were normal in number in most patients and moderately increased in several. The sedimentation rate was increased in sixteen patients. The atypical or abnormal lymphocytes which characterize the disease varied between 30 and 82 per cent. Moderate to marked toxic neutrophils with a moderate shift to the left in the neutrophilic picture was seen in all patients.

The heterophile antibody reaction was positive in thirteen patients, and negative in six; the test was not performed in the remaining six patients. A false-positive Wassermann reaction was not observed.

The bone marrow was hyperplastic in twenty patients and a quantitatively normal marrow was observed in five. The erythroid tissue was normal, except in the patient with the microcytic and hypochromic anemia in whom the bone marrow revealed a normoblastic hyperplasia. The megakaryocytes were normal or increased in number and showed a normal maturation dispersion. The myeloid elements besides being increased in number revealed a moderate to marked degree of granulopoietic immaturity. Immaturity was never carried to the stage of myeloblastic involvement. The atypical lymphocytes of the peripheral blood were conspicuously absent from the concentrated bone marrow material. The presence of a few atypical lymphocytes as well as a few normal lymphocytes, monocytes, and most of the mature granulocytes was due to sinusoidal dilution (peripheral blood). There is no evidence that the atypical lymphocytes which characterize infectious mononucleosis have their origin from cells in the bone marrow. The atypical lymphocytes lack the malignant characteristic that is seen in cases of leucemia, where the lymphocytes replace and crowd out the normal bone marrow elements.

One case of infectious mononucleosis (heterophile titer of 1:1792) was seen in a 25-year-old female with pulmonary tuberculosis. The leucocyte count

reached a peak of 15,500 with 69 per cent atypical lymphocytes. The bone marrow was in the hyperplastic group. The infectious mononucleosis cleared up, but the pulmonary tuberculosis became progressively worse and eventually required surgical therapy (thoracoplasty).

It is concluded that (1) in spite of the large number of atypical lymphocytes in the peripheral blood in infectious mononucleosis, the bone marrow is not involved; (2) the bone marrow reveals a myeloid hyperplasia and immaturity. Either the "toxic agent" acts on the bone marrow to produce a myeloid hyperplasia and suppresses their delivery to the peripheral blood or the suppression of the myeloid cells results in a myeloid hyperplasia; (3) the atypical lymphocytes in infectious mononucleosis show none of the metastatic or replacement characteristics of leucemic cells; (4) in contrast, in patients with acute, subacute, and chronic lymphatic leucemia with many abnormal lymphocytes in the peripheral blood the bone marrow reveals moderate to marked replacement of the normal bone marrow elements. The replacement characteristic of leucemic cells in lymphatic leucemia is seen in the bone marrow when the leucocyte count in the peripheral blood is leucopenic, normal, or increased in type; and (5) sternal puncture is of diagnostic aid in differentiating benign and malignant types of lymphocytosis.

### INFLUENZAL ENCEPHALITIS

G. O. BROWN, M.D., R. O. MUETHER, M.D., HENRY PINKERTON, M.D.  
(BY INVITATION), AND MARGARET LEGIER, B.S. (BY INVITATION)  
ST. LOUIS, MO.

Leichtenstern, in 1890, described an encephalitis believed due to influenza. Since discovery of influenza virus, no reports have appeared of its isolation from clinical cases of encephalitis. The case here reported was apparently caused by a virus resembling in some way influenza virus.

The patient, a white man, 51 years of age, became ill in January, 1944, when influenza was prevalent in St. Louis. His illness was characterized by fever, severe headache, somnolence, anorexia, nausea, and vomiting. There were few neurologic findings, practically no neck rigidity, and conjunctivitis and reddening of the pharynx were the chief physical findings. The patient had a respiratory illness, resembling mild influenza, six weeks prior to onset of headache.

Spinal fluid showed a cell count of 366 leucocytes and 100 erythrocytes per cubic millimeter. Leucocytes were practically all lymphocytes; protein, 160 mg. per 100 c.c.; Kahn and Wassermann tests, negative. Spinal fluid was bacteriologically sterile.

The patient's serum was tested for the titer of antibodies against influenza A and B virus employing the chicken cell agglutination test of Hirst. A titer of 1/1025 against influenza virus type A was found sixteen days after onset of illness, dropping to much lower levels two months later. The titer against influenza type B was much lower.

Spinal fluid was inoculated in eggs previously incubated for twelve days and subjected then to an additional forty-eight hours of incubation. The fluid,

yolk, and embryo of these eggs tested by the method of Hirst showed positive agglutination of chicken cells, a phenomenon suggesting the presence of influenza virus. The positive test persisted through seven series of egg transfers.

The material from the inoculated eggs, after the third transfer, produced pneumonia by intranasal inoculation in mice, the pathologic picture being similar to pneumonia produced by known influenza virus. Many of these pneumonic lungs were bacteriologically sterile. Intracerebral inoculation of this egg material produced a fatal illness in mice with pathologic evidence of a mild encephalitis.

The agglutination of chicken cells by this agent was not inhibited by known influenza type A or type B antiserum but was inhibited by the patient's own serum in a dilution of 1/250. This suggests a serologic difference between this agent and known strains of influenza virus. It resembles influenza virus in its ability to agglutinate chicken cells and in the pathologic lesions produced.

#### DISCUSSION

DR. THOMAS FRANCIS, JR., Ann Arbor, Mich.—I think it quite important to emphasize the reservation that has been made in the presentation of this evidence. Regardless of how many similar agents one can show, one cannot say it is the influenza virus. There are many things that might be equally attributed to mouse pneumonia virus. It has been reported by one group that mouse pneumonia virus will cause agglutination of mouse red cells.

In our experience with a large group of spinal fluids from individuals with mild neurologic disturbances at the time of illness, we were unable to get any evidence of an encephalitic virus being present. I think it is still important to accept this as encephalitis due to an undesignated virus until the evidence for influenza virus is more precise. From the abstract I think it is a question whether this might be called encephalitis clinically, since the signs are much more suggestive of meningitis than of true encephalitis.

DR. BROWN (closing).—We realize the difficulty which Doctor Francis suggests, namely, that there are viruses which occur spontaneously in mice which will cause pneumonia. We have gone back to the original egg material several times and succeeded in transmitting the disease to mice each time.

We are leaning very heavily on the Hirst chicken cell agglutination test as evidence for the presence of influenza virus. This test was positive in inoculated eggs prior to any mouse passage. Failure of known influenza A and B antiserum to inhibit chicken cell agglutination by this agent means that we cannot demonstrate its serologic identity with either of these strains of influenza virus. It might, however, be a serologically different strain.

I do not think that the clinical picture suggests meningitis more than encephalitis. Usually in meningitis there is a distinct neck rigidity and a higher spinal fluid cell count would be expected. The pathologic picture produced by this agent in the brains of inoculated mice was not that of a meningitis. Known influenza virus gave a pathologic picture both in brain and lungs of inoculated mice similar to that produced by this agent. I realize that these pathologic pictures are not too specific.

EPIDEMIOLOGIC OBSERVATIONS ON THE USE OF GLYCOL VAPORS  
FOR AIR STERILIZATION\*

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F. C. W. OLSON, B.S. (BY INVITATION)  
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The clearly established bactericidal and virucidal activity of glycol vapors on air-suspended microorganisms suggested the use of these materials as a new means for attack on the problem of control of air-borne infection. Studies on the engineering aspects of practical application were carried out, following which we were able to proceed with installation of apparatus for vapor generation and distribution at a military cantonment. This field trial was conducted to determine if the incidence of air-borne disease could be reduced by the treatment of living quarters with triethylene glycol vapor in effective concentration. Studies were made on three groups of 640 men, observed for six-week intervals and equally divided into test and control, the former sleeping in glycol treated quarters, the latter in untreated dormitories. We were able to demonstrate the following findings: (1) the apparatus maintained adequate glycol concentrations (0.0025-0.004 mg. per liter of air) and optimum relative humidity; (2) an over-all reduction in bacterial air contamination was obtained with practically complete abolition of hemolytic streptococci from the treated dormitories; (3) the incidence of positive throat cultures (hemolytic streptococci) fell sharply in the test groups at the end of the six-week observation period; there was evidence of prevention of spread of these organisms in the test quarters; (4) reduction in incidence of those diseases believed to be disseminated by the aerial route was effected; these infections included common cold, catarrhal fever, influenza, measles, mumps, scarlet fever, rheumatic fever, acute tonsillitis, chicken pox, acute sinusitis, and pneumonia. The over-all reduction for the full six-week period was 12 per cent. However, when the final seventeen days of each period was studied a reduction of 64 per cent was noted. We believe this to be due to two factors; one, the possible increase in length of incubation of respiratory infections brought about by the unusual conditions existing in military groups, and two, the lowering of infecting doses (lessening of air contamination) in the treated barracks as compared to the control, thus permitting those individuals with a moderate degree of immunity to escape disease.

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\*Work done under contract with the Office of Scientific Research and Development.

## HEREDITY OF THE Rh BLOOD TYPES

### IV. MEDICOLEGAL APPLICATION IN CASES OF DISPUTED PARENTAGE

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IN A preceding paper<sup>1</sup> data were presented on the Rh blood types in a series of ninety-seven families with 275 children. Only a single contradiction was found to Wiener's theory<sup>2</sup> of six major allelic genes, and in this case the child in question proved to be illegitimate. The original investigation did not yield sufficient data to test the theory adequately with regard to the rarer genes, *Rh'* and *Rh''*, but in a subsequent paper<sup>3</sup> a number of pedigrees were presented which included individuals bearing these rare genes. In the meantime, we have accumulated an additional series of families in which the bloods were tested for the Rh types, and the principal purpose of the present paper is to record these newer results.

In our first study on the heredity of the Rh blood types, we also reported twenty-three medicolegal cases in which tests were carried out for Rh types as well as for the A-B groups, A<sub>1</sub>-A<sub>2</sub> subgroups, and M-N types. In the present paper we propose to describe seventy-nine additional cases and to discuss the reliability of the Rh tests when applied in cases of disputed parentage.

#### MATERIALS AND METHODS

The blood samples tested in the heredity studies were obtained from two main sources: (1) families of acquaintances, technicians, and patients, and (2) families which were investigated because it was suspected that one or more of the children had congenital hemolytic disease (erythroblastosis fetalis). The latter accounts for the relatively large number of families with Rh-negative mothers. The medicolegal cases came from various courts in New York City, especially the Court of Special Sessions, and also included private cases and cases referred by courts outside of New York City.

The technique of performing the grouping, subgrouping, M-N, and Rh tests has been described in detail elsewhere<sup>4, 5</sup> and will not be repeated here. With regard to the nomenclature of the Rh types and Rh antisera, see Wiener.<sup>6, 7</sup>

A number of families was also examined for agglutinin P. The serum used in these tests came from a patient who had developed anti-P agglutinins as a result of repeated blood transfusions. This case is reported in detail in another paper<sup>8</sup> in which is also described the technique of performing the tests.

#### RESULTS

In Table I are presented the results of the grouping, subgrouping, M-N, and Rh tests in a series of 91 families with 161 children. In the first 24 families, tests were also carried out for agglutinin P.

From the Serological Laboratory of the Office of the Chief Medical Examiner of New York City and the Blood Transfusion Division of the Jewish Hospital of Brooklyn.

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TABLE I  
LIST OF FAMILY MATERIAL

	FAMILY		CHILDREN			
	FATHER	MOTHER				
1	A <sub>1</sub> MP+Rh <sub>1</sub>	A <sub>1</sub> MP-Rh <sub>1</sub> -	A <sub>1</sub> MP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
2	ONP+Rh <sub>1</sub> Rh <sub>2</sub>	OMP-Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
3	ONP+Rh <sub>1</sub>	ONP+Rh <sub>1</sub>	ONP+Rh <sub>1</sub> Rh <sub>2</sub> ♂	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
4	OMNP+Rh <sub>1</sub>	ONP+Rh <sub>1</sub> -	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
5	A <sub>1</sub> MNP-Rh <sub>1</sub>	A <sub>1</sub> MP+Rh <sub>1</sub> Rh <sub>2</sub>	OMP-Rh <sub>1</sub> Rh <sub>2</sub>	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
6	A <sub>2</sub> NP+Rh <sub>1</sub> Rh <sub>2</sub>	ONP+Rh <sub>1</sub>	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
7	OMNP+Rh <sub>1</sub>	A <sub>1</sub> NP-Rh <sub>1</sub>	ONP+Rh <sub>1</sub> Rh <sub>2</sub> ♀	ONP+Rh <sub>1</sub> Rh <sub>2</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
8	OMP+Rh <sub>2</sub>	OMP+Rh <sub>2</sub>	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> Rh <sub>2</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
9	ONP-Rh <sub>1</sub>	OMP+Rh <sub>2</sub>	OMP+Rh <sub>2</sub> ♂	OMNP+Rh <sub>1</sub> ♀	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
10	ONP-Rh <sub>1</sub> Rh <sub>2</sub>	OMNP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> ♂	OMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
11	A <sub>1</sub> MP-Rh <sub>1</sub> Rh <sub>2</sub>	OMNP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> ♂	OMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
12	A <sub>1</sub> MP-Rh <sub>1</sub>	A <sub>1</sub> MP-Rh <sub>1</sub> Rh <sub>2</sub>	OMNP+Rh <sub>1</sub> ♀	OMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
13	OMNP+Rh <sub>1</sub> -	A <sub>1</sub> MP-Rh <sub>2</sub>	A <sub>1</sub> MP-Rh <sub>1</sub> ♂	OMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
14	ONP+Rh <sub>1</sub> Rh <sub>2</sub>	AMNP-Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> ♀	A <sub>1</sub> MP-Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
15	A <sub>1</sub> MNP+Rh <sub>2</sub>	A <sub>1</sub> MP+Rh <sub>1</sub>	A <sub>2</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
16	OMP+Rh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MP+Rh <sub>1</sub> -	A <sub>1</sub> MP+Rh <sub>2</sub> ♀	A <sub>1</sub> MP+Rh <sub>2</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
17	OMNP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> -	A <sub>1</sub> MP+Rh <sub>2</sub> ♀	A <sub>1</sub> MP+Rh <sub>2</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
18	ONP+Rh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MP+Rh <sub>1</sub>	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP-Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
19	A <sub>1</sub> MP-Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> -	A <sub>1</sub> MP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
20	ONP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> -	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
21	A <sub>1</sub> MNP+Rh <sub>1</sub>	ONP+Rh <sub>1</sub> -	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
22	OMNP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> -	OMNP+Rh <sub>1</sub> ♀	OMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
23	OMP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> ♂	OMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
24	OMP+Rh <sub>1</sub> -	A <sub>1</sub> BMNP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> ♂	OMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
25	A <sub>1</sub> MNP+Rh <sub>1</sub>	BMNP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
26	BMNP+Rh <sub>1</sub>	BMNP+Rh <sub>1</sub>	BMNP+Rh <sub>1</sub> ♂	BMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
27	A <sub>1</sub> MNP+Rh <sub>2</sub>	BMNP+Rh <sub>1</sub>	BMNP+Rh <sub>1</sub> ♂	BMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
28	A <sub>1</sub> MNP+Rh <sub>2</sub>	BMNP+Rh <sub>1</sub>	BMNP+Rh <sub>1</sub> ♂	BMNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
29	A <sub>1</sub> Rh <sub>1</sub>	OMNP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> ♀	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
30	A <sub>2</sub> BMNP+Rh <sub>2</sub>	ONP+Rh <sub>1</sub> -	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
31	A <sub>1</sub> MNP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> -	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
32	A <sub>1</sub> MNP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> -	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
33	BMNP+Rh <sub>1</sub>	A <sub>1</sub> BMNP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
34	ONP+Rh <sub>1</sub>	A <sub>1</sub> BMNP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
35	OMNP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> -	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
36	BMNP+Rh <sub>1</sub>	A <sub>1</sub> BMNP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
37	ONP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
38	OMNP+Rh <sub>1</sub>	BMNP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
39	ONP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
40	OMNP+Rh <sub>1</sub>	OMNP+Rh <sub>1</sub> -	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
41	BMNP+Rh <sub>1</sub>	BMNP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂
42	BMNP+Rh <sub>1</sub>	BMNP+Rh <sub>1</sub>	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	A <sub>1</sub> MNP+Rh <sub>1</sub> ♂	ONP+Rh <sub>1</sub> ♂	A <sub>1</sub> MP+Rh <sub>1</sub> ♂

Negro families.		Triplets.		Newborn twins.	
A <sub>1</sub> MRh <sub>2</sub>	A <sub>1</sub> MRh <sub>1</sub> -	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ †	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ
A <sub>1</sub> MRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub> -	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀
A <sub>1</sub> MNRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MNRh <sub>1</sub> -	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ
A <sub>1</sub> MNRh <sub>1</sub> -	BMNRh <sub>1</sub>	BMNRh <sub>1</sub>	BMNRh <sub>1</sub>	BMNRh <sub>1</sub>	BMNRh <sub>1</sub>
A <sub>1</sub> MNRh <sub>2</sub>	A <sub>1</sub> MNRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ
A <sub>1</sub> MNRh <sub>1</sub>	OMNRh <sub>1</sub> -	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀
A <sub>1</sub> MRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub>
AMRh <sub>1</sub> -	BMRh <sub>1</sub>	BMRh <sub>1</sub>	BMRh <sub>1</sub>	BMRh <sub>1</sub>	BMRh <sub>1</sub>
BMNRh <sub>1</sub>	BMRh <sub>1</sub>	BMRh <sub>1</sub>	BMRh <sub>1</sub>	BMRh <sub>1</sub>	BMRh <sub>1</sub>
A <sub>1</sub> MRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MRh <sub>1</sub> -	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ
A <sub>1</sub> MRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ
ONRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ
ANRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub> -	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ
A <sub>1</sub> MNRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀
OMRh <sub>1</sub> Rh <sub>2</sub>	BMNRh <sub>1</sub> -	BMNRh <sub>1</sub> δ	BMNRh <sub>1</sub> δ	BMNRh <sub>1</sub> δ	BMNRh <sub>1</sub> δ
OMRh <sub>1</sub>	OMNRh <sub>1</sub> -	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ
A <sub>1</sub> MRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ
A <sub>1</sub> MNRh <sub>1</sub> -	BMNRh <sub>1</sub>	BMNRh <sub>1</sub>	BMNRh <sub>1</sub>	BMNRh <sub>1</sub>	BMNRh <sub>1</sub>
A <sub>1</sub> MRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MRh <sub>1</sub> -	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ	A <sub>1</sub> MRh <sub>1</sub> δ
A <sub>1</sub> MRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀
ONRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub> -	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ
OMRh <sub>1</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ
A <sub>1</sub> BMNRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ
BMRh <sub>1</sub> -	ANRh <sub>1</sub>	ANRh <sub>1</sub> δ	ANRh <sub>1</sub> δ	ANRh <sub>1</sub> δ	ANRh <sub>1</sub> δ
OMNRh <sub>1</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ
A <sub>1</sub> NRh <sub>2</sub>	A <sub>1</sub> NRh <sub>1</sub>	A <sub>1</sub> NRh <sub>1</sub> δ	A <sub>1</sub> NRh <sub>1</sub> δ	A <sub>1</sub> NRh <sub>1</sub> δ	A <sub>1</sub> NRh <sub>1</sub> δ
OMRh <sub>1</sub>	OMRh <sub>1</sub>	OMRh <sub>1</sub> δ	OMRh <sub>1</sub> δ	OMRh <sub>1</sub> δ	OMRh <sub>1</sub> δ
OMNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ	A <sub>1</sub> MNRh <sub>1</sub> δ
OMRh <sub>1</sub>	BMRh <sub>1</sub> -	BMRh <sub>1</sub> δ	BMRh <sub>1</sub> δ	BMRh <sub>1</sub> δ	BMRh <sub>1</sub> δ
A <sub>1</sub> NRh <sub>1</sub>	ANRh <sub>1</sub> -	ANRh <sub>1</sub> δ	ANRh <sub>1</sub> δ	ANRh <sub>1</sub> δ	ANRh <sub>1</sub> δ
A <sub>1</sub> MRh <sub>1</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ
A <sub>1</sub> NRh <sub>2</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀	OMNRh <sub>1</sub> ♀
OMNRh <sub>1</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ	OMNRh <sub>1</sub> δ
OMRh <sub>1</sub>	A <sub>1</sub> BMNRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> BMNRh <sub>1</sub>	A <sub>1</sub> BMNRh <sub>1</sub>	A <sub>1</sub> BMNRh <sub>1</sub>	A <sub>1</sub> BMNRh <sub>1</sub>

•Negro families.

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†Triplet

197-10-1000

In Table II, the hereditary transmission of the Rh types in the ninety-one families is summarized.\* With only a single apparent exception (Family 46), the results conform with the theory that the eight Rh types are transmitted by a series of six allelic genes,  $Rh_1$  (or  $Rh_o'$ ),  $Rh_2$  (or  $Rh_o''$ ),  $Rh'$ ,  $Rh''$ ,  $Rh_o$ , and  $rh$ . In view of the importance of the apparent exception to the theory, this case will be described in detail.

TABLE II  
SUMMARY OF FAMILY MATERIAL OF TABLE I

MATING	NUMBER OF FAMILIES	NUMBER OF CHILDREN OF TYPES						
		Rh-	$Rh_1$	$Rh_2$	$Rh_1Rh_2$	$Rh_o$	$Rh'$	TOTALS
Neg. $\times$ Neg.	2	5	0	0	0	0	0	5
Neg. $\times$ $Rh_1$	32	12	36	0	0	4	0	52
Neg. $\times$ $Rh_2$	10	9	0	13	0	0	0	22
Neg. $\times$ $Rh_1Rh_2$	11	(1)	10	6	0	0	0	17
Neg. $\times$ $Rh_o$	2	0	0	0	0	2	0	2
$Rh_1 \times Rh_1$	10	1	16	0	0	0	0	17
$Rh_1 \times Rh_2$	7	2	3	1	6	0	1	13
$Rh_1 \times Rh_1Rh_2$	5	0	10	0	1	0	0	11
$Rh_1 \times Rh_o$	1	1	0	0	0	0	0	1
$Rh_1 \times Rh'$	2	0	2	0	0	0	0	2
$Rh_1 \times Rh''$	1	0	0	0	1	0	0	1
$Rh_2 \times Rh_2$	1	0	0	3	0	0	0	3
$Rh_2 \times Rh_1Rh_2$	1	0	1	3	4	0	0	8
$Rh_1Rh_2 \times Rh_1Rh_2$	4	0	1	1	3	0	0	5
$Rh_1Rh_2 \times Rh_o$	1	0	1	0	0	0	0	1
$Rh_1Rh_2 \times Rh'$	1	0	0	0	1	0	0	1
Totals	91	31	80	27	16	6	1	161

The mother of Family 46 was admitted in labor to the obstetric ward of Bellevue Hospital with the following history. Her first pregnancy had resulted in a normal male child who was 6 years old and well, while the second had resulted in a normal female child who was 4 years old and well. Neither of these two children was available for examination. The patient's third pregnancy had terminated two and one-half years ago with a stillborn male infant. At that time the woman was found to be Rh negative with strong anti-Rh agglutinins in her serum, indicating that the stillbirth was a manifestation of hemolytic disease. The present pregnancy was her fourth one. Tests were carried out on patient and husband and it was found that the husband belonged to type  $Rh_1Rh_2$ . It therefore appeared certain that the expected infant would have hemolytic disease because it had to be Rh positive. Much to everyone's surprise, the newborn infant proved to be normal in every respect. Serologic tests then showed the child to be Rh negative which explained why it had escaped the disease, but this raised a new problem because this finding was contrary to the expectations under the genetic theory. When the patient was confronted with these facts she practically admitted that her husband was not the father of the infant.

No contradictions were encountered to the laws of inheritance of the blood groups, subgroups or M-N types in any of the families. Inasmuch as only a few studies have been carried out to date on the heredity of the agglutinin P, our results in twenty-four families have been summarized in Table III. It will be seen that our findings conform with the theory that the agglutinin P is transmitted as a simple Mendelian dominant.<sup>10</sup>

In Table IV, we have summarized the data from our pedigrees illustrating the transmission of the rare gene  $Rh'$  and  $Rh''$  which were reported in a previous paper as already mentioned. In Table V we have combined the data of Tables II and IV with the family data presented in our first genetic study of the Rh blood types. It will be seen that to date we have determined the

\*In two families (2 and 18) there were individuals with blood giving intermediate reactions.<sup>9</sup> For the sake of simplicity, data on these individuals are included in Table II together with the data on the type  $Rh_1$  individuals.



TABLE III  
HEREDITY OF AGGLUTINOGEN P IN FAMILIES OF TABLE I

MATING	NUMBER OF FAMILIES	CHILDREN		
		P+	P-	TOTALS
P+ × P+	13	29	3	32
P+ × P-	9	19	5	24
P- × P-	2	0	4	4
Totals	24	48	12	60

TABLE IV  
SUMMARY OF FAMILY MATERIAL FROM STUDY OF SONN AND WIENER

MATING	NUMBER OF FAMILIES	NUMBER OF CHILDREN OF TYPES							
		Rh-	Rh <sub>1</sub>	Rh <sub>2</sub>	Rh <sub>1</sub> Rh <sub>2</sub>	Rh <sub>0</sub>	Rh'	Rh''	TOTALS
Neg. × Rh'	1	1	0	0	0	0	1	0	2
Neg. × Rh''	1	1	0	0	0	0	0	5	6
Rh <sub>1</sub> × Rh <sub>2</sub>	1	0	1	0	0	0	0	0	1
Rh <sub>1</sub> × Rh <sub>2</sub> Rh <sub>2</sub>	1	0	6	2	2	0	0	0	10
Rh <sub>1</sub> × Rh'	1	0	1	0	0	0	0	0	1
Rh <sub>1</sub> × Rh''	3	1	2	0	2	0	0	0	5
Rh <sub>2</sub> × Rh'	1	0	0	0	0	1	1	0	2
Totals	9	3	10	2	4	1	2	5	27

TABLE V  
SUMMARY OF AUTHORS' FAMILY MATERIAL TO DATE

MATING	NUMBER OF FAMILIES	NUMBER OF CHILDREN OF TYPES							
		Rh-	Rh <sub>1</sub>	Rh <sub>2</sub>	Rh <sub>1</sub> Rh <sub>2</sub>	Rh <sub>0</sub>	Rh'	Rh''	TOTALS
Neg. × Neg.	4	14	0	0	0	0	0	0	14
Neg. × Rh <sub>1</sub>	49	25	73	0	0	7	0	0	105
Neg. × Rh <sub>2</sub>	16	10	0	20	0	0	0	0	30
Neg. × Rh <sub>1</sub> Rh <sub>2</sub>	15	(1)	18	14	0	0	0	0	33
Neg. × Rh <sub>0</sub>	3	3	0	0	0	3	0	0	6
Neg. × Rh'	2	1	0	(1)	0	0	1	0	3
Neg. × Rh''	1	1	0	0	0	0	0	5	6
Rh <sub>1</sub> × Rh <sub>1</sub>	26	5	62	0	0	4	1	0	72
Rh <sub>1</sub> × Rh <sub>2</sub>	21	6	15	7	18	0	2	0	48
Rh <sub>1</sub> × Rh <sub>1</sub> Rh <sub>2</sub>	27	0	46	8	25	0	0	0	79
Rh <sub>1</sub> × Rh <sub>0</sub>	1	1	0	0	0	0	0	0	1
Rh <sub>1</sub> × Rh'	3	0	3	0	0	0	0	0	3
Rh <sub>1</sub> × Rh''	4	1	2	0	3	0	0	0	6
Rh <sub>2</sub> × Rh <sub>2</sub>	2	1	0	4	0	0	0	0	5
Rh <sub>2</sub> × Rh <sub>1</sub> Rh <sub>2</sub>	6	0	2	8	8	0	0	0	18
Rh <sub>2</sub> × Rh <sub>0</sub>	2	0	0	1	0	4	0	0	5
Rh <sub>1</sub> Rh <sub>2</sub> × Rh <sub>1</sub> Rh <sub>2</sub>	10	0	3	1	17	0	0	0	21
Rh <sub>1</sub> Rh <sub>2</sub> × Rh <sub>0</sub>	2	0	1	1	0	0	0	0	2
Rh <sub>1</sub> Rh <sub>2</sub> × Rh'	2	0	1	1	2	0	0	0	4
Rh <sub>0</sub> × Rh'	1	0	0	0	0	1	1	0	2
Totals	197	69	226	66	73	19	5	5	463

Rh blood types in 197 families with 463 children. In the entire series, there were only two apparent exceptions to the theory of six allelic genes (indicated by the parentheses in Table V), both of which have been readily explained because the children in question were shown to be illegitimate.

#### MEDICOLEGAL APPLICATIONS

Our family studies on the Rh blood types as well as statistical studies on the distribution of the types in the general population<sup>11</sup> have proved the accuracy of the theory of six allelic genes beyond any reasonable doubt. Moreover

TABLE VI  
RESULTS OF TESTS IN CASES OF DISPUTED PARENTAGE

CASE	PUTATIVE FATHER	MOTHER	CHILDREN	INTERPRETATION
1*	A <sub>1</sub> MNRh <sub>0</sub>	A <sub>2</sub> NRh <sub>0</sub>	A <sub>1</sub> MNRh <sub>0</sub> ♂	No exclusion
2	OMRh <sub>2</sub>	OMNRh'	OMNRh- ♂	No exclusion
3	A <sub>1</sub> MNRh <sub>1</sub>		A <sub>1</sub> MNRh <sub>1</sub> ♀	No exclusion
4	ONRh <sub>1</sub> Rh <sub>2</sub>	OMRh <sub>1</sub>	a) OMRh <sub>1</sub> ♂ b) OMNRh <sub>1</sub> Rh <sub>2</sub> ♂ c) OMNRh <sub>1</sub> Rh <sub>2</sub> ♂	None of the children excluded
5*	A <sub>2</sub> NRh <sub>0</sub>	OMNRh <sub>2</sub>	A <sub>1</sub> MNRh <sub>2</sub> ♂	No exclusion
6	A <sub>1</sub> BMNRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub>	BMRh <sub>1</sub> Rh <sub>2</sub> ♀	No exclusion
7	A <sub>2</sub> MNRh <sub>1</sub>	BMNRh <sub>2</sub>	A <sub>2</sub> BMNRh <sub>2</sub> ♀	No exclusion
8	A <sub>2</sub> NRh <sub>2</sub>	A <sub>1</sub> NRh <sub>1</sub>	OMNRh <sub>1</sub> ♀	Excluded by M-N tests
9†	BMNRh <sub>2</sub>	BMNRh-	BMRh <sub>0</sub>	No exclusion
10	A <sub>1</sub> BMNRh <sub>1</sub>	OMNRh <sub>1</sub>	a) BMNRh <sub>1</sub> ♀ b) BMNRh <sub>1</sub> ♂	Neither child excluded
11	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub> ♀	No exclusion
12	OMRh <sub>1</sub>	BNRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MNRh <sub>2</sub> ♂	Exclusion by A-B-O tests
13	OMNRh <sub>1</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> ♂	No exclusion
14	a) OMRh <sub>1</sub> b) A <sub>2</sub> MNRh <sub>1</sub>	OMRh <sub>1</sub>	OMRh <sub>1</sub> ♀	Neither man excluded
15	A <sub>2</sub> MNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> NRh <sub>1</sub> Rh <sub>2</sub> ♂	No exclusion
16	BMRh <sub>1</sub>	OMRh <sub>1</sub>	OMRh <sub>1</sub> ♀	No exclusion
17	A <sub>1</sub> MRh <sub>1</sub>	A <sub>2</sub> BMNRh <sub>1</sub>	a) BMRh <sub>1</sub> ♂ b) BNRh <sub>1</sub> ♂	Second child excluded by M-N tests
18	A <sub>1</sub> NRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub>	OMRh <sub>1</sub> ♂	Excluded by M-N tests
19	BMNRh <sub>1</sub>	OMNRh-	BMNRh <sub>1</sub> ♂	No exclusion
20*	OMNRh <sub>0</sub>	A <sub>2</sub> NRh <sub>2</sub>	A <sub>1</sub> MNRh <sub>0</sub> ♀	No exclusion
21	OMRh <sub>1</sub> Rh <sub>2</sub>	BMNRh <sub>1</sub>	OMNRh <sub>1</sub> Rh <sub>2</sub> ♀	No exclusion
22	BNRh-	OMRh <sub>2</sub>	OMNRh- ♂	No exclusion
23†	OMNRh <sub>1</sub>	BMRh <sub>1</sub>	BMRh <sub>1</sub> ♂	No exclusion
24†	BMNRh <sub>1</sub>	ONRh-	a) BMNRh <sub>1</sub> ♀ b) BMNRh- ♂	Neither child excluded
25	A <sub>1</sub> MNRh <sub>1</sub>	OMNRh-	A <sub>1</sub> MRh <sub>1</sub> ♂	No exclusion
26*	OMRh <sub>0</sub>	A <sub>1</sub> NRh <sub>0</sub>	A <sub>1</sub> MNRh <sub>0</sub> ♂	No exclusion
27‡	A <sub>1</sub> BMNRh <sub>1</sub>	BNRh-	BNRh- ♂	No exclusion
28	OMRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub> ♀	No exclusion
29	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> NRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MNRh <sub>2</sub> ♂	No exclusion
30	A <sub>1</sub> BNRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub> ♀	No exclusion
31†	A <sub>1</sub> MRh <sub>1</sub>	BNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub> ♂	No exclusion
32†	OMRh <sub>1</sub> Rh <sub>2</sub>	A <sub>2</sub> MRh <sub>2</sub>	A <sub>1</sub> MRh <sub>2</sub> ♀	Exclusion by A <sub>1</sub> -A <sub>2</sub>
33*	A <sub>2</sub> NRh <sub>1</sub> Rh <sub>2</sub>		A <sub>1</sub> BMRh <sub>0</sub> ♂	Triple exclusion by A <sub>1</sub> -A <sub>2</sub> , M-N, and Rh tests
34	ONRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>2</sub>	A <sub>1</sub> MNRh- ♂	Exclusion by M-N tests
35	A <sub>2</sub> MNRh <sub>1</sub>	OMRh <sub>1</sub>	a) OMNRh <sub>1</sub> ♀ b) OMNRh- ♂	Neither child excluded
36*	OMRh <sub>1</sub>	BMNRh <sub>1</sub>	BMRh <sub>1</sub> ♀	No exclusion
37	OMNRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub> ♂	A <sub>1</sub> MRh <sub>1</sub> ♂	No exclusion
38	ONRh-	BMNRh <sub>1</sub>	BNRh <sub>1</sub> ♀	No exclusion
39	A <sub>1</sub> NRh-	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> NRh <sub>1</sub> ♂	No exclusion
40†	A <sub>1</sub> MRh <sub>1</sub>	OMNRh <sub>1</sub>	OMRh <sub>1</sub>	No exclusion
41†	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> NRh <sub>1</sub>	No exclusion
42	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub> Rh <sub>2</sub> ♀	Exclusion by Rh tests
43	A <sub>1</sub> MRh <sub>1</sub> Rh <sub>2</sub>	OMRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub> ♂	No exclusion
44	OMNRh <sub>1</sub>	A <sub>1</sub> MRh-	A <sub>1</sub> MRh <sub>1</sub> ♀	No exclusion
45	A <sub>1</sub> MRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub> Rh <sub>2</sub> ♀	No exclusion
46	OMNRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub>	OMNRh <sub>2</sub> ♀	No exclusion
47*	A <sub>1</sub> NRh-	ONRh <sub>0</sub>	ONRh <sub>1</sub> ♂	No exclusion
48	OMRh <sub>2</sub>	BMNRh <sub>1</sub> Rh <sub>2</sub>	BMRh <sub>1</sub> Rh <sub>2</sub> ♀	No exclusion
49	OMNRh <sub>0</sub>	OMNRh-	OMNRh- ♀	No exclusion

\*Negro.

†Race not known; blood samples received by mail.

‡Man, Chinese; woman, Negro.

§Man, Negro; woman, white.

||M-N tests on baby not reliable because of blood transfusions.

¶Man, American Indian; woman, Negro.

\*\*Twins.

TABLE VI—CONT'D

CASE	PUTATIVE FATHER	MOTHER	CHILDREN	INTERPRETATION
50	BMRh <sub>1</sub>	OMRh <sub>2</sub>	BMRh <sub>1</sub> ♀	No exclusion
51	OMNRh <sub>2</sub>	OMNRh <sub>2</sub>	ONRh <sub>1</sub> ♂	No exclusion
52§	A <sub>1</sub> MNRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub>	OMNRh <sub>2</sub> ♀	No exclusion
53	A <sub>1</sub> MNRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub> Rh <sub>2</sub>	ORh <sub>1</sub> Rh <sub>2</sub> ♂	No exclusion
54*	A <sub>1</sub> MNRh <sub>2</sub>	BMNRh <sub>2</sub>	A <sub>1</sub> BMNRh <sub>1</sub> ♂	No exclusion
55	A <sub>1</sub> NRh <sub>1</sub> —	OMRh <sub>1</sub>	OMNRh <sub>1</sub> ♀	No exclusion
56	OMNRh <sub>1</sub> —	A <sub>1</sub> NRh <sub>1</sub>	ONRh <sub>1</sub> ♂	No exclusion
57	OMNRh <sub>1</sub>	ONRh <sub>1</sub> —	OMNRh <sub>1</sub> ♂	No exclusion
58	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> —	OMNRh <sub>1</sub> ♂	No exclusion
59*	OMNRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MRh <sub>1</sub> ♀	No exclusion
60	A <sub>2</sub> BMNRh <sub>1</sub>		A <sub>2</sub> BNRh <sub>1</sub> ♀	No exclusion
61	A <sub>1</sub> MRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub> ♀	No exclusion
62*	BMRh <sub>2</sub>	A <sub>1</sub> NRh <sub>2</sub>	A <sub>1</sub> MNRh <sub>2</sub> ♂	No exclusion
63*	A <sub>1</sub> NRh <sub>1</sub>	A <sub>2</sub> MRh <sub>1</sub>	A <sub>2</sub> MRh <sub>1</sub> Rh <sub>2</sub> ♂	Double exclusion by M-N and Rh tests
64	A <sub>1</sub> MRh <sub>1</sub>	A <sub>2</sub> MNRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub> ♂	No exclusion
65*	OMNRh <sub>1</sub> —	OMNRh <sub>2</sub>	a) OMNRh <sub>2</sub> ♀ b) OMNRh <sub>2</sub>	Neither child excluded
66	BMRh <sub>1</sub>	OMRh <sub>1</sub>	BMRh <sub>1</sub> ♀	No exclusion
67*	OMRh <sub>2</sub>	OMNRh <sub>2</sub>	BMNRh <sub>1</sub> ♂	Exclusion by A-B-O tests
68†	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub>	No exclusion
69	BNRh <sub>1</sub>	ONRh <sub>1</sub>	BNRh <sub>1</sub> Rh <sub>2</sub> ♀	No exclusion
70	OMNRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MNRh <sub>1</sub> Rh <sub>2</sub> ♀	No exclusion
71	BMNRh <sub>1</sub>	A <sub>1</sub> MRh <sub>1</sub>	BMRh <sub>1</sub> ♂	No exclusion
72	OMNRh <sub>1</sub> Rh <sub>2</sub>	OMNRh <sub>1</sub> Rh <sub>2</sub>	A <sub>2</sub> NRh <sub>1</sub> ♂	Exclusion by A-B-O tests
73†	OMNRh <sub>2</sub>	OMRh <sub>2</sub>	OMNRh <sub>2</sub> ♂	No exclusion
74	BMNRh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> MNRh <sub>1</sub>	A <sub>1</sub> BMNRh <sub>1</sub> Rh <sub>2</sub> ♂	No exclusion
75*	A <sub>2</sub> NRh <sub>2</sub>	OMNRh <sub>1</sub>	a) ONRh <sub>2</sub> ♂ b) A <sub>2</sub> MNRh <sub>2</sub> ♀	Neither child excluded
76	OMNRh <sub>1</sub>	OMNRh <sub>1</sub>	OMNRh <sub>1</sub> ♀	No exclusion
77*	BMNRh <sub>1</sub> Rh <sub>2</sub>	A <sub>2</sub> BMNRh <sub>1</sub>	BMNRh <sub>1</sub> ♀	No exclusion
78*	ONRh <sub>1</sub>	BMNRh <sub>2</sub>	a) A <sub>2</sub> BMNRh <sub>2</sub> ♂ ** b) BMRh <sub>2</sub> ♂ **	First child excluded by A-B tests; second child excluded by M-N tests
79	A <sub>1</sub> MNRh <sub>1</sub> —	A <sub>1</sub> NRh <sub>1</sub>	A <sub>1</sub> MNRh <sub>1</sub> ♂	No exclusion

Race and co-workers,<sup>12</sup> working independently in England, have collected data in a series of fifty-six families with ninety children, all conforming with the expectations under the theory. Therefore, the medicolegal application of the Rh blood types in cases of disputed parentage is justified and, as has already been mentioned, for the past eighteen months we have carried out Rh typings routinely together with the grouping, subgrouping, and M-N tests in all cases of disputed parentage. In Table VI, seventy-nine new cases of disputed paternity are listed which, together with our previously reported series of twenty-three, bring our total beyond the 100 mark.

The application of the Rh types for the exclusion of paternity is based on the following laws of inheritance:

1. The factors Rh<sub>0</sub> and Rh' and Rh'' are transmitted as simple Mendelian dominants and therefore cannot appear in the blood of a child unless present in the blood of one or both parents. In applying this law it must be borne in mind that while there are three Rh factors, there are five agglutinogens, Rh<sub>0</sub>, Rh', Rh<sub>1</sub>, Rh'', and Rh<sub>2</sub>, where Rh<sub>1</sub> contains the factors Rh<sub>0</sub> and Rh' together and Rh<sub>2</sub> contains the factors Rh<sub>0</sub> and Rh'' together. While agglutinogens Rh<sub>1</sub> and Rh<sub>2</sub> are usually transmitted as units by the corresponding genes Rh<sub>1</sub>, and Rh<sub>2</sub>, in occasional individuals (genotype Rh<sub>0</sub>Rh' and Rh<sub>0</sub>Rh'') the factors will exist as distinct agglutinogens that segregate genetically. Therefore, while agglutinogens Rh<sub>1</sub> and Rh<sub>2</sub> will usually behave as if they were simple Mendelian dominants, there will be rare families where the child will belong to type Rh<sub>1</sub> (or type Rh<sub>2</sub>) and yet neither parent will have the agglutinin Rh<sub>1</sub>

(or  $Rh_2$ ). For example, in the rare mating  $Rh_0 \times Rh'$ , usually one-fourth of the children will give reactions corresponding to type  $Rh_1$ .

2. Parents belonging to type  $Rh_1Rh_2$  or the rare type  $Rh'Rh''$  cannot have children belonging to type  $Rh_0$  or Rh negative. Similarly, parents of types  $Rh_0$  and Rh negative cannot have children of type  $Rh_1Rh_2$  or  $Rh'Rh''$ . This law is obvious because parents of types  $Rh_1Rh_2$  and  $Rh'Rh''$  must transmit one of the genes  $Rh_1$ ,  $Rh_2$ ,  $Rh'$ , or  $Rh''$  to each child, while parents of types  $Rh_0$  and Rh negative can transmit only either an  $Rh_0$  or  $rh$  gene to each child.

3. Further exclusions of parentage are possible when more than one child is available for testing. For example, in the mating  $Rh_1 \times Rh$  negative, if there is one Rh-negative child, then the  $Rh_1$  parent must belong to genotype  $Rh_1rh$ , and children belonging to any type other than  $Rh_1$  or Rh negative will be excluded. On the other hand, in the mating  $Rh_1 \times Rh$  negative, if there are any children of types  $Rh_0$  or  $Rh'$ , there can be no Rh-negative children. This law will rarely be applicable in medicolegal cases because these usually involve only a single child.

The value of the Rh tests in cases of disputed paternity can be gleaned from the fact that whereas previously, with the aid of the A-B-O and M-N tests, a falsely accused man had one chance in three of proving his innocence, the Rh tests have raised the chances of exclusion to approximately 45 per cent. In the present series, the accused man was excluded by the Rh tests in three cases. In one case (63) the man was also excluded by the M-N tests. The second case (33) is of interest because even though the mother's blood was not available for testing, the accused individual, a young Negro lad, was excluded by three separate tests. He belonged to subgroup  $A_2$  and the child to subgroup  $A_1B$ ; he belonged to type N and the child to type M; and he belonged to type  $Rh_1Rh_2$  and the child to type  $Rh_0$ . In the third case (42), the man was excluded by the Rh tests where the other tests were inconclusive. Together with our previously reported exclusion (Case 3, Wiener, Sonn, and Belkin<sup>1</sup>) there were two cases among our total of 102 cases, in which the man was excluded by the Rh tests, but the woman's false accusation might have been believed if we had had to depend only on the A-B-O and M-N tests.

It may be of some interest to point out that there was one exclusion involving the subgroups of A (Case 32). In another interesting case (25) the blood of the putative father, who belonged to group A, gave intermediate reactions with the absorbed B serum; the mother belonged to group O and the child to subgroup  $A_1$ . The difference in reactivity between the blood of the putative father and child with the absorbed B serum was not considered evidence of nonpaternity because of the limited knowledge concerning the hereditary transmission of the intermediate subgroups of A.

#### OTHER APPLICATIONS OF THE Rh BLOOD TYPES

The Rh blood types are also useful for the individual identification of fresh, wet bloodstains. The common blood groups and subgroups give rise to six types of blood, the agglutinogens M and N determine three types of blood, with anti-P serum two types of blood can be distinguished, while with the three varieties of anti-Rh sera and with anti-Hr serum,<sup>7, 13</sup> eight types of blood can be distinguished (disregarding the rare types  $Rh'Rh''$  and homozygous  $Rh'$ ), so that in all  $6 \times 3 \times 2 \times 8 = 288$  varieties of human blood can readily be differentiated.

In a previous paper<sup>14</sup> it was shown that the Rh genes are not sex-linked and most likely are carried in a different pair of chromosomes from the *A-B-O* and *M-N* genes. The discovery of the existence of six major allelic genes in the Rh series has materially increased the value of these tests for linkage studies in human genetics.

A third application is in relation to the problem of superfecundation. If an Rh-negative mother gave birth to triplets each belonging to a different Rh type, this could only be explained by assuming that at least two of the triplets had different fathers.

#### COMMENT

In this paper, for the sake of simplicity, we have confined the presentation to the six standard genes and the eight Rh types which they determine. No attempt has been made to discuss the Hr factor or Race and Taylor's<sup>15</sup> rare *Rh<sub>u</sub>* and *Rh<sub>z</sub>* genes or the rare intermediate genes described by Wiener.<sup>8</sup> The existence of these rare genes complicates the heredity mechanism, making it necessary to apply the second law of inheritance with some reservation, although no qualification is necessary for exclusions of paternity based on the first law.

With regard to the nomenclature of the Rh genes and types, at first a numbered system was used by us, but the present nomenclature was substituted as soon as the serology and genetics were worked out. Murray's<sup>16</sup> contention that the discovery by Race and Taylor of their *Rh<sub>u</sub>* gene makes necessary a total revision of the nomenclature is fallacious, because the present designations can easily be extended to include any additional genes that may possibly be encountered (cf. Wiener<sup>9</sup>). Incidentally, there was no need to change the names of the groups and subgroups or the M-N types when the rare genes *A<sub>1</sub>* and *N<sub>1</sub>* were discovered. Aside from the fact that Murray's designations do not take cognizance of the special serologic and genetic positions of the anti-Rh<sub>0</sub> and anti-Hr sera in relation to anti-Rh' and anti-Rh'', they introduced two hypothetical sera which most likely do not even exist. Finally, it hardly seems a simplification to call blood of type Rh<sub>1</sub>Rh<sub>2</sub> "135-123," while Rh-negative blood is designated by Murray as "456."

#### SUMMARY

Data have been presented on the Rh types in a series of ninety-one families with 161 children. A single apparent exception to the theory of six allelic genes has been shown to be due to illegitimacy. These data combined with previously published results make a total of 197 families with 463 children tested by us for the Rh blood types. The satisfactory agreement between the observations and expectations under the theory justify the medicolegal application of the Rh tests for the exclusion of paternity.

To date, in 102 cases of disputed paternity, tests for the Rh types have been carried out together with the usual A-B-O, *A<sub>1</sub>-A<sub>2</sub>*, and M-N tests. In four cases the accused man was excluded by the Rh tests; in two of these cases he was also excluded by one of the other tests, but in two cases the woman's false accusation might have been believed had the Rh tests not been performed.

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## THE RELATIONSHIP BETWEEN CHANGES IN SERUM PROTEIN CONCENTRATION AND THE PLASMA VOLUME IN NORMAL SUBJECTS

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### INTRODUCTION

CHANGES in the concentration of the serum proteins have been used as an index of alterations in the volume of the plasma. This is based on the concept that, in the absence of capillary damage, the amount of protein in the blood stream remains constant. The demonstration by Madden and Whipple,<sup>1</sup> that serum protein is in a state of "dynamic equilibrium" and may be added to or taken from the blood stream with ease, would suggest that the plasma protein concentration would not necessarily be a good index of increases or decreases in the plasma volume.

This is a report of a comparison between induced changes in the plasma volume and the accompanying variations in the total protein concentration and the total circulating protein in normal subjects. It indicates that the changes in serum protein concentration fail to reflect proportionate alterations in the plasma volume because, under these circumstances, serum protein may be added to or taken from the blood stream.

### METHODS AND MATERIAL

The subjects were normal medical students or ambulatory hospital patients, in good health at the time of the observations, who had been on a routine hospital diet for some time. As far as could be determined at the time of the studies, they were in a good state of nutrition and normal hydration. None had had previous edema or cardiovascular or renal disease.

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Increases in the plasma volume greater than 5 per cent were produced in forty-four instances by the daily oral administration of 20 Gm. of sodium bicarbonate, or 25 Gm. of sodium chloride,<sup>2</sup> the administration of desoxycorticosterone acetate intramuscularly,<sup>3</sup> or the ingestion of large amounts of water. Decreases in the plasma volume greater than 5 per cent were produced on forty-one occasions by the administration of ammonium chloride,<sup>4</sup> mercupurin,<sup>5</sup> or both, and by simple dehydration.

Plasma volume determinations were made by the method of Gibson and Evelyn.<sup>6</sup> Fifteen to twenty-five minutes were allowed for the mixing of the dye and subsequently six samples were taken under oil at five- to six-minute intervals. The serum protein concentration was determined by the falling drop method of Barbour and Hamilton<sup>7</sup> on the serum drawn for the plasma volume.

All plasma volume determinations were made in the morning with the patient supine in the rested postabsorptive state. Following the initial determination, the subject was placed on a regime affecting the water and salt content of the body and the determinations repeated twenty-four, forty-eight, or seventy-two hours later under the same conditions. The subjects receiving only large amounts of water remained in the rested fasting state but had the determinations repeated in from three to five hours.

From the product of the plasma volume and the concentration of serum protein per 100 c.c. of serum, the total amount of protein present in the serum may be calculated. Since the plasma volume varies with the size of the individual, the extent of alterations in the plasma volume, serum protein concentration, and total circulating protein are for the purpose of comparison expressed as percentage change from the initial determination. The relationship between alterations in these three factors has been studied by a comparison of the mean percentage change with increases and decreases in the plasma volume, the coefficient of correlation, and by a comparison of the individual changes to the standard error of estimate from a linear regression for increased and decreased plasma volume and for the two groups combined.

The determinations of the plasma volume, serum protein concentration, and total circulating protein remain quite constant from day to day in normal subjects following their usual daily routine. In Table I is shown the reliability of the determinations in control subjects on whom observations were repeated on successive mornings.

TABLE I

CHANGES FOUND IN CONTROL SUBJECTS WITH DETERMINATIONS MADE ON SUCCESSIVE MORNINGS

	NUMBER OF PAIRED OBSERVA- TIONS	MEAN PER- CENTAGE CHANGE (PER CENT)	STANDARD DEVIATION OF MEAN (PER CENT)	STANDARD ERROR OF MEAN	PROBABLE INCIDENCE OF CHANGES GREATER THAN +5 PER CENT	PROBABLE INCIDENCE OF CHANGES GREATER THAN -5 PER CENT
Plasma volume	22	+0.68	2.80	0.61	0.07	0.02
Total serum protein concentration	13	-0.60	3.49	1.0	0.06	0.11
Total circulating protein	13	+0.29	3.71	1.07	0.10	0.08
Hematocrit	22	-0.79	2.63	0.56	0.02	0.06
Red blood cell vol- ume	22	-2.32	4.78	1.04	0.06	0.30

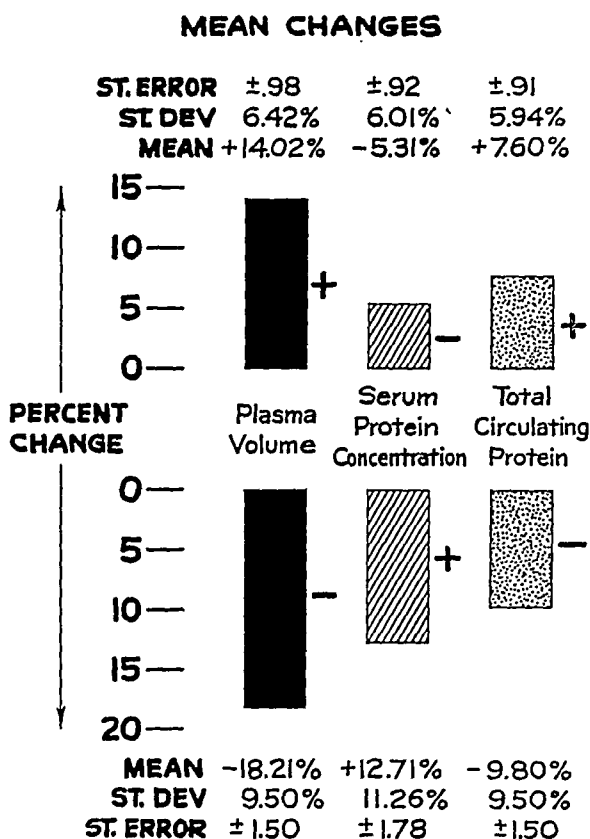


Fig. 1.—Mean percentage change in plasma volume, serum protein concentration, and total circulating protein in normal subjects with induced increases and decreases in plasma volume.

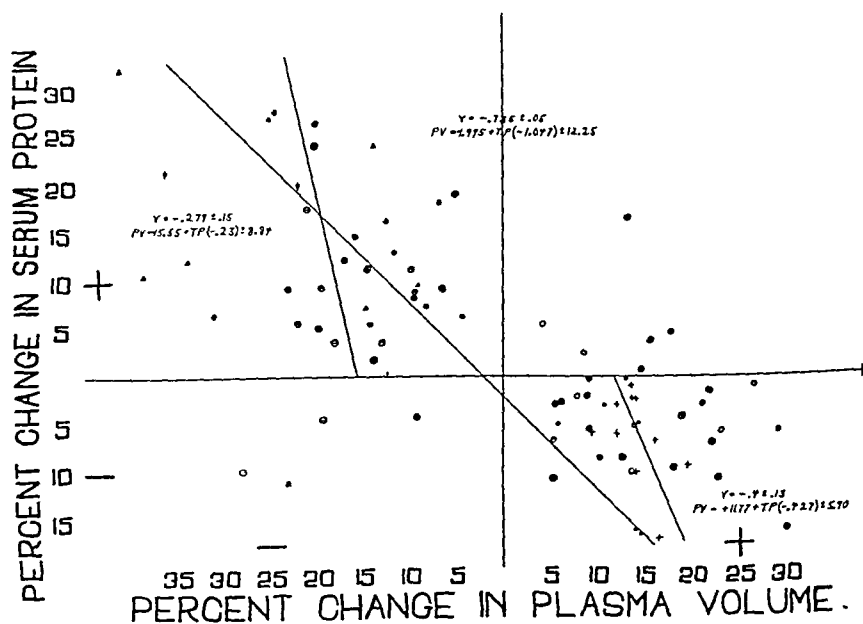


Fig. 2.—Individual variations in the percentage change of serum protein concentration and plasma volume and their relation to a linear regression.



## RESULTS

The mean percentage change in the plasma volume, serum protein concentration, and total circulating protein for each group of observations are shown in Fig. 1. The total circulating protein was altered in the direction of the shift in plasma volume. The mean percentage change in total circulating protein represented about 54 per cent of the mean percentage variation in the plasma volume in both groups. The serum protein concentration failed to reflect the extent of the alteration in the plasma volume. This was especially true with increases in the plasma volume where the mean percentage change in serum protein concentration represented only 37.8 per cent of the mean alteration in the plasma volume.

The variation in the relative changes in the plasma volume and serum protein concentration may be better visualized by a graph of the individual observations (Fig. 2). It will be noted that with decreases of plasma volume, the serum protein concentration changed considerably, with large inconsistent variations. Increases in the plasma volume, on the other hand, were associated with small decreases in the concentration of serum protein which, in all but four of the subjects, changed less than 10 per cent. It is apparent from an inspection of this graph that a linear relationship between percentage change in plasma volume and serum protein concentration for both groups may be described only with considerable error.

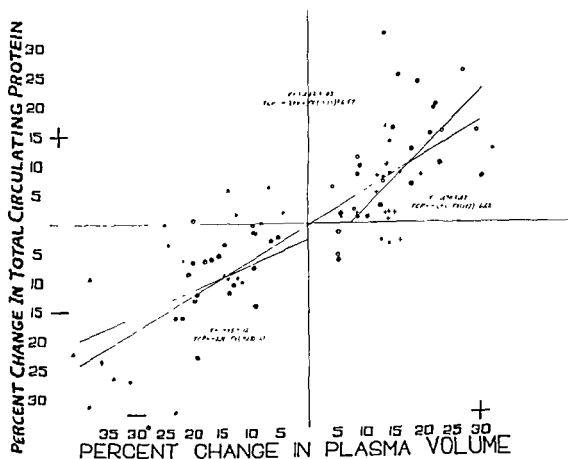


Fig. 3.—Individual variations in plasma volume and total circulating protein and their relation to a linear regression.

The coefficient of correlation between the relative variation in plasma volume and serum protein concentration was satisfactory when both groups were combined but of no significance in either group alone, indicating that there appears to be only a directional change in serum protein concentration with change in plasma volume. A prediction of the change in plasma volume directly from change in serum protein concentration would not only be subject to considerable

killed bacteria cells could be coated with antigen and that such antigen-coated cells made possible the detection of extremely small amounts of antibody. Their studies have been limited to virus diseases and so far as can be determined, the principles they described have not been applied to other antigens. It was decided to attempt to coat bacteria with tuberculin for the purpose of developing a quantitative precipitin test against tuberculin.

#### PROCEDURE

*Preparation of Bacterial Suspension.*—*Serratia marcescens* were used because of their small uniform size. One cubic centimeter of a twenty-four hour broth culture of the bacteria was seeded onto plain agar in a Kolle flask and incubated for twenty-four hours. It was then washed off with physiologic saline adjusted to a pH of 6. The bacterial suspension was centrifuged and washed twice with the physiologic saline. After the final washing the bacteria were suspended in 20 c.c. of neutral distilled water and killed with flowing steam. This is the stock cell suspension and will keep indefinitely if stored in a refrigerator at 8° C.

*Preparation of Old Tuberculin Solution.*—Standard commercial old tuberculin solution (Mulford) was used. The tuberculin was diluted to 0.5 per cent by placing 0.5 c.c. of the old tuberculin in a 100 c.c. sterile volumetric flask and diluting to the mark with sterile physiologic saline. This solution is used as a stock solution and will keep for months if stored in the refrigerator.

*Preparation of the Antigen.*—The nitrogen content of the stock cell suspension and of the 0.5 per cent old tuberculin solution was determined by acid and superoxol digestion and nesslerization. The cell suspension was diluted so that the nitrogen content was the same as that of the dilute old tuberculin. Equal portions of each, that is, dilute cell suspension and 0.5 per cent old tuberculin, were mixed and incubated at 37.5° C. for twelve hours. The old tuberculin was adsorbed on the cells in this interval. The mixture was then centrifuged and the coated cells thrown down. These were washed twice in saline to remove any excess or free old tuberculin. The coated cells were finally suspended in physiologic saline (pH 6) to a density equivalent to a reading of 70 on the Klett-Summerson photoelectric colorimeter, using a green filter (540 mμ). This suspension was used as the antigen for the test.

*Test.*—The test proper was done as follows:

Blood from the subject was obtained in the usual manner, observing aseptic precautions. The blood was placed in a sterile tube and, after clotting, the serum was carefully removed. It may be stored in the cold indefinitely if free of contamination.

Serial dilutions were made of the serum ranging from 1:8 to 1:2,048 or more. Then 0.5 c.c. of antigen was added to 1 c.c. of each of the serum dilutions. The tubes were shaken vigorously for five minutes (Kahn shaker may be used) and incubated at 37.5° C. for two hours. The test was read at this time, then placed in a refrigerator at 8° C. for twelve hours, and reread after being allowed to warm up to room temperature.

Positive tests were manifested by a fine granular precipitate best read against a blue light in a darkened room.

Suitable controls were carried out using untreated cells and diluted serum antigen and saline controls. During the early stages of the study a control was carried out in all dilutions, but in later work

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## PRECIPITIN TEST FOR TUBERCULIN ANTIBODIES\*

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THE use of tuberculin in the diagnosis of tuberculosis has not been too satisfactory since it is not, strictly speaking, a quantitative test, and the amount of antibody which develops in the patient cannot be followed easily. The complement fixation test, too, has not been satisfactory.

In 1940, Cannon and Marshall<sup>1</sup> showed that the sensitivity of the precipitin test can be enhanced by the use of antigen-coated particles, and Weir<sup>2</sup> applied this technique to the demonstration of antibodies against tuberculin. However, the difficulties inherent in the production of uniform collodion particles make this test technically difficult. In 1941, Roberts and Jones<sup>3</sup> demonstrated that

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It would appear that it is not the change in the plasma volume per se that controls the quantity of circulating protein but rather the alteration in the concentration of the serum protein so that the osmotic equilibrium between the plasma and the extracellular fluid is not greatly disturbed by large changes in the plasma volume.

It was evident in these subjects that the concentration of serum protein paralleled the decreases in plasma volume associated with dehydration more closely than with increases in plasma volume. With the loss of extracellular fluid in dehydration there is also a decrease in the tissue pressure with its resultant effect on the capillary osmotic equilibrium. Thus a relatively greater concentration of serum protein would be required to maintain the depleted plasma volume. It is also possible that these normal subjects are less able to remove readily excess protein from the blood stream than are hypoproteinemic subjects. On the other hand, with retention of water and salt the increased volume of the extracellular fluid may play a role in maintaining the elevated plasma volume and the concentration of serum protein might be expected to fall considerably. However, it would appear that sufficient protein is added to the plasma so that the osmotic equilibrium between plasma and interstitial fluid is not greatly altered and the added protein also serves to maintain the elevated plasma volume.

Because of the change in the total circulating protein, prediction of the plasma volume based on variations in the concentration of serum protein will underestimate the plasma volume. In Fig. 4 are illustrated such predictions of the mean percentage change in the plasma volume based on the percentage change in serum protein concentration (Column 1), on the ratio of the initial plasma volume to the alteration in serum protein concentration (Column 2), and on the line of regression for these cases (Column 3) compared to the observed change for these cases (Column 3) and compared to the observed change in the plasma volume (Column 4).

Although approximation of the change in the plasma volume may be made from variation in the serum protein concentration, it should be remembered that such approximations tend to underestimate the changes, especially when the plasma volume is increased. The error of such predictions is sufficiently large, however, to suggest that the use of alteration in the serum protein concentration in evaluating changes in plasma volume in experimental observations is not justified, especially in instances where the change in plasma volume is relatively small.

#### SUMMARY

1. A comparison has been made between changes in the plasma volume, serum protein concentration, and total circulating protein in eighty-five studies on normal subjects.
2. The serum protein concentration is not altered in proportion to the change in the plasma volume due to the fact that protein is added to the plasma with increases in plasma volume or taken from the plasma with decreases in the plasma volume.
3. Prediction of the plasma volume from alteration in serum protein concentration tends to underestimate the degree of change in plasma volume and is associated with considerable error.

We wish to express appreciation to Drs. F. N. Wilson, H. C. Carver, and P. S. Dwyer, for their patient assistance in the statistical arrangement of the data.

only the 1:8 serum dilution was used. These two control tubes, namely, coated cells and saline and uncoated cells and serum, should always be included for each test.

It is imperative that the following precautions be observed:

1. Antigen, serum, and saline must be sterile.
2. Antigen, that is, coated cells, must be prepared daily or be kept frozen in a deep-freeze refrigerator at  $-20^{\circ}\text{C}$ .
3. The test must not be left in the refrigerator for more than twenty-four hours or false positive tests will result.
4. The precipitate is extremely fine and requires great care and considerable skill for accurate reading.

#### RESULTS

*Studies on Guinea Pigs.*—Twelve guinea pigs, after having been bled from the heart for control test, were inoculated into the liver with concentrated material known to contain tubercle bacilli. Two other guinea pigs were used for controls throughout the experiment. All animals were bled at weekly intervals after the preliminary or control bleeding.

The results are given in Table I. The preliminary bleeding and guinea pig controls were negative throughout.

TABLE I  
DEVELOPMENT OF AGGLUTININ AGAINST TUBERCULIN IN EXPERIMENTALLY INFECTED GUINEA PIGS

GUINEA PIG	CONTROL	FIRST WEEK	SECOND WEEK	THIRD WEEK
1	Neg.	Neg.	1:4	1:4
2	Neg.	Neg.	Neg.	Neg.
3	Neg.	Neg.	1:2	1:2
4	Neg.	1:4	1:8	1:16
5	Neg.	1:2	1:4	1:16
6	Neg.	Died	---	---
7	Neg.	1:128	1:8	1:32
8	Neg.	1:16	1:32	1:32
9	Neg.	1:8	1:16	1:32
10	Neg.	1:32	1:32	1:128
11	Neg.	1:8	1:64	Died
12	Neg.	1:16	1:64	1:128
Control	Neg.	Neg.	Neg.	Neg.
Control	Neg.	Neg.	Neg.	Neg.

One animal of the twelve injected failed to develop antibodies and one animal died during the first week of the experiment. The other ten developed antibodies but the titers varied from 1:2 to 1:128. It is possible that the low titers were the result of the overwhelming infection. This point is now under study.

*Studies on Tuberculin-Negative and Tuberculin-Positive Patients.*—A group of individuals were skin tested with old tuberculin and a sample of blood drawn from each for agglutination test. The blood was drawn before the skin test so that there could be no question of the antibody titer being disturbed by the old tuberculin.

The sera of eight tuberculin-positive and eight tuberculin-negative patients were then subjected to the agglutination test with results given in Table II.

It will be noted that only one tuberculin-negative individual had antibodies in the serum while all of the tuberculin-positive individuals had positive tests, although the titer was not high in any of these, ranging from 1:16 to 1:64.



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## THE SEROLOGIC DIAGNOSIS OF ENDEMIC TYPHUS

## II. A COMPARISON OF WATER-BATH AND ICEBOX FIXATION IN THE COMPLEMENT FIXATION TEST

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*Introduction.*—Complement fixation has been utilized in the study of a number of rickettsial diseases; for example, Rocky Mountain spotted fever,<sup>1</sup> Tobia fever of Colombia (Rocky Mountain spotted fever), "Q" fever of North America and Australia,<sup>2</sup> and endemic and epidemic typhus.<sup>3-6</sup> The reaction has been studied especially by Bengtson<sup>3</sup> in an attempt to determine its usefulness in the diagnosis of endemic typhus in man and by Brigham and Bengtson<sup>7</sup> in a comparison of various tests as means of diagnosis of the disease in experimentally and naturally infected rats. From the work of Bengtson it was concluded that the test was useful "in detecting recent and also past infection with endemic typhus virus" and that "the sensitivity of the test is indicated by the results with sera from cases in which infection occurred as long as nine years ago and as recently as seven days." Likewise, the studies of Brigham and Bengtson indicated that "the complement fixation reaction as applied to rat control programs should be a valuable aid in preventing the spread of endemic typhus to human beings."

The value of any serologic procedure as an aid in diagnosis depends on the two factors of specificity and sensitivity. Obviously, it matters little how sensitive a test is if it lacks specificity, and at the same time, its usefulness is limited if its sensitivity is so low that it fails to give reactions except with relatively high titer serum.

*The Test.*—The technique of the complement fixation test in rickettsial diseases has been described by Bengtson.<sup>8</sup> The reagents include guinea pig complement, anti-sheep cell rabbit hemolysin, and sheep red cells, antigen, and test serum. The antigen is a suspension of rickettsiae grown in the yolk sac of fertile hen eggs, following the method of Cox,<sup>9</sup> but this specially prepared suspension is not essential, as Damon and Johnson<sup>10</sup> have shown that commercial typhus vaccines are satisfactory substitutes for it.

Sera are inactivated for one-half hour at 56° C. and dilutions made in two-fold steps ranging from 1:4 to 1:512 or higher, if necessary, to reach the end point. Two-tenths cubic centimeter of serum with 0.2 c.c. of the proper dilution

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of antigen (containing 4 units) and 0.2 c.c. of complement (2 units) are combined and incubated for one hour at 37° C. in a water bath. Subsequently, 0.4 c.c. of a 2 per cent sensitized sheep cell suspension (containing 2 units of hemolysin in 0.2 c.c.) is added. The tests are then reincubated for one hour in the 37° C. water bath, placed in the cold room overnight, and read the following morning. The degrees of fixation are recorded as 4+, 3+, 2+, 1+, and trace, and the titer is considered to be the highest dilution showing 3+ or 4+ fixation. Serum, antigen, and hemolytic system controls are always included.

With the technique described, the results previously reported<sup>10</sup> were obtained.

*Purpose of This Study.*—The purpose of the present study was to extend the observations previously reported<sup>10</sup> by making a comparison of the results obtained when fixation was carried out at 37° C. in a water bath for one hour and at 6 to 8° C. overnight in the cold room. Our conclusions are based on the end points reached in determining antigenic titers of specially prepared rickettsial suspensions, commercial vaccines, diagnostic human serum from cases of suspected typhus, and rat sera collected in connection with the typhus control program.

*Results.*—The end points reached in the titration of specially prepared rickettsial suspensions and vaccines, when fixation was carried out in the water bath and the cold room, are shown in Table I.

From Table I it is evident that it made no difference whether the product was a specially prepared rickettsial suspension or a commercial vaccine; the antigenic titer was always higher by icebox fixation. The significance of this

TABLE I

ANTIGENIC TITERS OBTAINED WITH SPECIALLY PREPARED RICKETTSIAL ANTIGENS AND VACCINES WHEN FIXATION WAS ACCOMPLISHED BY ONE-HOUR INCUBATION IN THE WATER BATH AT 37° C. AND OVERNIGHT IN THE COLD ROOM

SOURCE OF ANTIGEN	STRAIN OF RICK-ETTSIA	ANTIGEN DILUTION												
		37° FIXATION						ICEBOX FIXATION						
		1:4	1:8	1:16	1:32	1:64	1:128	1:4	1:8	1:16	1:32	1:64	1:128	1:256
National Institute of Health	Endemic, Strain S2	4	4	4	2	-	-	4	4	4	4	4	2	-
	Endemic, Strain S4	4	4	4	4	-	-	4	4	4	4	4	2	-
Parke, Davis & Co. Vaccine														
792	Epidemic	4	4	1	-	-	-	4	4	4	3	-	-	-
793	Epidemic	4	4	3	-	-	-	4	4	4	4	2+	-	-
794	Epidemic	4	3	-	-	-	-	4	4	4	2	-	-	-
795	Epidemic	4	4	2	-	-	-	4	4	4	4	-	-	-
Lederle Laboratories, Inc. Vaccine														
719	Epidemic	4	4	3	-	-	-	4	4	4	4	2-	-	-
721	Epidemic	4	4	4	1	-	-	4	4	4	4	4	1	-
725	Epidemic	4	4	3-	-	-	-	4	4	4	4	-	-	-
726	Epidemic	4	4	-	-	-	-	4	4	4	1	-	-	-
739	Epidemic	4	4	2	-	-	-	4	4	4	4	-	-	-
43	Epidemic	4	4	3	-	-	-	4	4	4	4	2-	-	-
45	Epidemic	4	4	3	-	-	-	4	4	4	4	2-	-	-
50	Epidemic	4	4	1	-	-	-	4	4	4	4	-	-	-



TABLE II

TITERS OBTAINED WITH PATIENTS' SERA WHEN EXAMINED BY THE WEIL-FELIX, WATER-BATH AND ICEBOX COMPLEMENT FIXATION TESTS

DATE	PATIENT	WEIL-FELIX END TITER	SERUM DILUTION															
			37° C. FIXATION								ICEBOX FIXATION							
			1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
2/22/44	?	4+ 1,280	4	4	4	4	4	4	tr	-	4	4	4	4	4	4	4	2
2/28/44		4+ 160	4	4	4	4	4	4	tr	-	4	4	4	4	4	4	4	-
1/ 3/45		4+ 320	4	4	2+	4	4	4	-	-	4	4	4	4	4	4	4	-
1/ 1/45		4	4	4	4	4	4	4	1	-	-	4	4	4	4	4	4	-
1/ 6/45		4	4	4	4	4	4	4	4	-	-	4	4	4	4	4	4	-
1/17/45	W. E. K.	4+ 160	4	4	4	1	4	4	-	-	-	4	4	4	4	4	4	-
1/20/45	F 5021	4+ 2,560	4	4	4	4	4	4	4	3	-	4	4	4	4	4	4	3
1/23/45	F. G.	4+ 640	4	4	4	4	4	4	4	-	-	4	4	4	4	4	4	-
1/ 2/45	D. W.	4+ 40	4	4	4	4	4	4	4	4	-	4	4	4	4	4	4	-
1/13/45	D. W.	4+ 2,560	4	4	4	4	4	4	4	4	-	4	4	4	4	4	4	Trace
1/11/45	W. W. Y.	4+ 80	4	4	4	4	4	4	4	4	-	4	4	4	4	4	4	-
1/15/45	W. W. Y.	4+ 1,280	4	4	4	3	4	4	-	-	-	4	4	4	4	4	4	-

TABLE III

RESULTS OBTAINED WITH RAT SERA IN COMPLEMENT FIXATION TESTS CARRIED OUT AT WATER BATH AND ICEBOX TEMPERATURE

LAB. NO.	SERUM DILUTION															
	37° C. FIXATION								ICEBOX FIXATION							
	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
76	-	4	4	4	-	-	-	-	4	4	4	4	4	4	4	-
2	4	4	4	4	4	-	-	-	4	4	4	4	4	4	4	2
3	4	4	4	4	4	-	-	-	4	4	4	4	4	4	4	2
8	4	4	4	4	4	-	-	-	4	4	4	4	4	4	4	-
15	1	4	4	4	4	-	-	-	4	4	4	4	4	4	4	-
19	4	4	4	4	4	-	-	-	4	4	4	4	4	4	4	-
21	4	4	4	4	4	-	-	-	4	4	4	4	4	4	4	-
22	4	4	4	4	4	-	-	-	4	4	4	4	4	4	4	-
23	4	4	4	4	4	-	-	-	4	4	4	4	4	4	4	-
25	1	4	4	4	4	-	-	-	4	4	4	4	4	4	4	-
32	4	4	4	4	4	-	-	-	4	4	4	4	4	4	4	-
39	-	4	4	4	4	-	-	-	4	4	4	4	4	4	4	-

observation lies in the fact that by using this technique a greater dilution of the antigen may be made and for a given number of tests a smaller amount of the undiluted antigen will be required.

In Table II are shown the results obtained when diagnostic human sera were titrated by both procedures. As a matter of interest, the Weil-Felix end titers are included, together with the complement fixation results in two instances where second specimens were requested. In every instance it is obvious that the icebox test is more sensitive, as indicated by the degree of fixation obtained or the dilution of patients' sera in which a reaction was observed. In the two cases cited in which second specimens were submitted by the doctor, this difference in sensitivity is especially noticeable. Both of these sera were completely negative on first examination when tested by the water-bath procedure, but both gave a suspicious reaction in the icebox test. The validity of these suspicious tests is shown by the results when the second specimens were examined, as findings in both patients were positive in high dilutions of the serum. Such results are, of course, highly significant in a diagnostic laboratory (Table II).

Finally, there is to be considered the matter of the examination of blood from wild rats, as this is especially important in epidemiologic and control studies. The use of the Weil-Felix reaction in this connection has been reviewed by Brigham and Bengtson,<sup>7</sup> who concluded from their own work that the test was, at most, infrequently positive in the areas studied. On the other hand, they remarked that the complement fixation test "as applied to rat control programs should be a valuable aid in preventing the spread of endemic typhus to human beings." With this in mind, then, it is highly important to use the most sensitive technique available, and the results we have obtained with rat sera using water-bath and icebox fixation are shown in Table III.

As was the case with human sera, the results from rat sera indicate in all instances a stronger reaction in the tests when fixation was accomplished at icebox temperature. By this technique it also appears that in certain specimens a degree of reaction may be anticipated, although the same sera are completely negative when tested at water-bath temperature.

#### CONCLUSIONS

With the technique now recommended, comparative complement fixation tests, carried out at water-bath and icebox temperatures, yield results definitely in favor of the latter. In some instances the titers of the sera are considerably higher, in other instances some degree of reaction is obtained though parallel tests at water-bath temperature are completely negative, and in all instances the titers have been at least equal to those obtained at 37° C. This greater sensitivity of the icebox technique is especially important in a diagnostic laboratory in detecting sera having minimal concentrations of antibodies as shown in the cases cited where second specimens showed markedly rising titers.

In our experience no difficulty with anticomplementary reactions has been encountered except in sera that were old or possibly contaminated, and the controls have always been satisfactory. It is not our intention to imply, however, that the technique cannot be improved, and studies now in progress are directed toward that end. It is our belief, however, that in a diagnostic laboratory complement fixation for the detection of endemic typhus infection is best carried out at icebox temperature.

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## COMPARISON OF IN VITRO AND IN VIVO PENICILLIN RESISTANCE OF A STRAIN OF HEMOLYTIC STAPHYLOCOCCUS AUREUS

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TESTS in vitro<sup>1</sup> to determine the sensitivity to penicillin of organisms cultured from patients with infections have been useful in the selection of cases suitable for treatment with penicillin and in the regulation of the course of treatment. The concentration of penicillin in the blood of patients injected intramuscularly every three hours with 25,000 units of this substance seldom rises above 0.1 units per cubic centimeter. Immediately after the administration, intravenously, of 10,000 units of penicillin, the level reaches little more than 1.0 unit per cubic centimeter. Tests in vitro with concentrations of penicillin in these ranges usually give evidence that the organisms being tested are either sensitive or resistant to the levels of penicillin in the blood of patients being treated.

Recently a strain of hemolytic *Staphylococcus aureus* which was resistant in vitro to concentrations of penicillin below 10 units per cubic centimeter was isolated from a patient with an abscess resulting from osteomyelitis of bones of the orbit. Penicillin was administered intramuscularly and the abscess was drained by surgical incision. The patient recovered promptly although cultures remained positive for the strain of hemolytic *Staphylococcus aureus* until the drainage from the wound ceased. The efficacy of penicillin in aiding the recovery of this patient, and the reliability of the sensitivity test in vitro were questioned. Further confirmation of resistance to penicillin of this strain of

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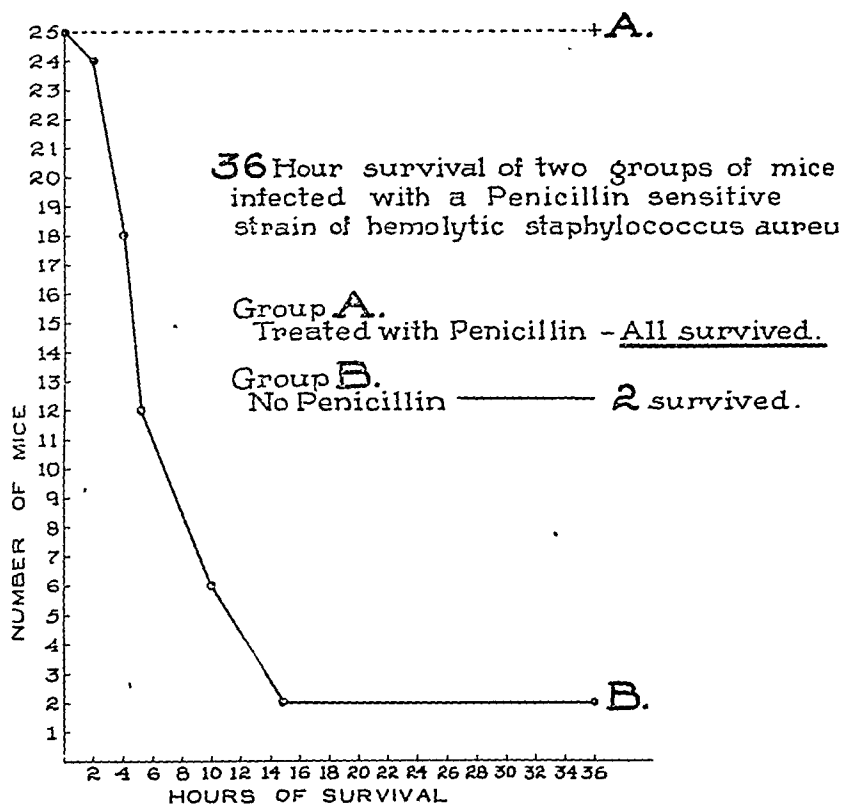


Fig. 1.

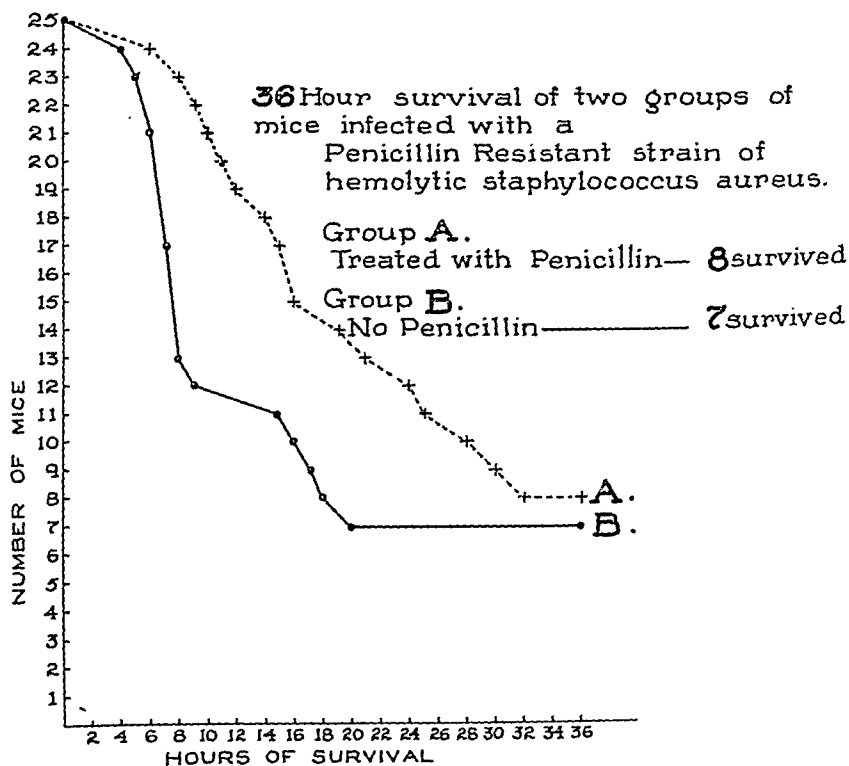


Fig. 2.

hemolytic *Staphylococcus aureus* was sought by comparing its resistance to penicillin in vitro with experiments in vivo in mice.

Mice excrete penicillin more rapidly than human beings do, and it was found necessary to inject 200 units of penicillin per gram of body weight every one and one-half to two hours in order to obtain a level of about 1 unit of penicillin per cubic centimeter of blood. The mice varied from 10 to 30 grams in weight, the average being 15 grams. Each animal received 3,000 units of penicillin dissolved in 1 c.c. of physiologic saline intraperitoneally every one and one-half hours for the first four and one-half hours and less frequently thereafter as the infected mice developed edema. The remaining injections were given at intervals of from three to six hours, averaging about four hours. Half an hour after the third injection of 3,000 units the level of penicillin was 4.48 units per cubic centimeter of blood, three and one-half hours after the fifth injection it was zero. Rammelkamp's method<sup>2</sup> was used to measure the levels of penicillin in the blood.

For this experiment the cultures of hemolytic *Staphylococcus aureus* were grown for twenty-four hours on agar slants, washed off, and measured in Hopkins' tubes. Since the pathogenicity of staphylococci varies considerably in different strains, it was necessary to determine the dose that would be lethal to mice in about twenty-four hours. Ten billion *Staphylococcus aureus* organisms of this strain inoculated intraperitoneally were found to be the minimum lethal dose that would kill most of the mice in approximately twenty-four hours.

Each of fifty mice was inoculated intraperitoneally with 10 billion staphylococci of the strain resistant to penicillin; each of fifty others was inoculated with a similar dose of a strain of hemolytic *Staphylococcus aureus* sensitive to penicillin. Twenty-five mice in each group were injected with penicillin. The results are summarized in Figs. 1 and 2.

#### SUMMARY

In experiments using mice, a strain of hemolytic *Staphylococcus aureus* that was resistant to penicillin in vitro proved also to be resistant to comparable concentrations of penicillin in vivo.

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## FURTHER STUDIES ON CHOLINE DEFICIENCY IN DOGS

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**S**TUDIES on choline deficiency in young puppies have been reported from this laboratory.<sup>1, 2</sup> The principal lesion observed in this deficiency was an extensive fatty metamorphosis of the liver and was accompanied by marked impairment of liver function as measured by bromsulfalein elimination, plasma phosphatase, plasma cholesterol and cholesterol ester levels, and prothrombin time. These liver function tests are not specific for this particular condition but reflect parenchymal liver disease of various etiology. If more specific tests for choline deficiency were available, it would be of interest to apply them to patients with liver disease. Since an excess of methionine protects the pup from this deficiency, the primary defect is thought to be one of "labile methyl" rather than of choline per se.<sup>1</sup> It would appear that a method of testing the "methyl reserves" of the deficient and control animals might serve as a more specific index of the state of nutrition with respect to choline. The role of labile methyl from methionine or choline in the synthesis of creatine and creatinine in the rat, rabbit, and man has been summarized by du Vigneaud.<sup>3</sup> He has suggested that other nitrogen or sulfur-linked substances containing methyl groups present in tissues are dependent on dietary choline or methionine for their formation. Among these we have selected the N-methylated derivatives of nicotinic acid for study along with creatinine and creatine. In this paper we wish to present some observations on the excretion of these substances by choline-deficient and control puppies together with observations on improvement in liver function in choline deficiency following choline therapy.

### EXPERIMENTAL\*

Two litters of three puppies each were used in these experiments. All were given Ration 5<sup>1</sup> but containing 3 per cent cod-liver oil instead of 2 per cent and 1 per cent less sucrose. In addition, 20 mg. per cent of nicotinic acid was added to the ration of Dogs 38, 40, 41, and 42 in order to insure greater excretion of methylated derivatives of nicotinic acid. One pup in each of the two litters (Dogs 39 and 42) was given a supplement of 200 mg. of choline chloride per 100 Gm. of ration; the other two in each litter received only the basal ration. The dogs were all weighed and given water-soluble vitamin supplements orally each day and injections of Pitman-Moore antidistemper serum weekly as previously described.<sup>1</sup> Food consumption records were kept daily. The growth curves of these animals are given in Fig. 1. Dogs 38, 40, and 41 were given choline supplements after a suitable period on the deficient ration, and the return to normal liver function was observed by carrying out liver function tests every three or four days. Dog 38 was sacrificed on the tenth day after beginning the choline supplement, and the total lipids of the liver of this animal were determined. Liver function tests were obtained by methods previously described.<sup>1</sup>

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The dogs were housed in metabolism cages during the periods of study of the excretion of creatine, creatinine, and N methyl nicotinic acid derivatives. The daily urinary excretion of these substances was determined from suitable aliquots of filtered urine. Creatinine was determined colorimetrically using the alkaline picrate color measured in the Coleman spectrophotometer at 500  $m\mu$ . Creatine was determined in the same fashion after conversion to creatinine by autoclaving with 2N sulfuric acid. Total urinary derivatives of N methyl nicotinic acid were determined by the method of Sarett.<sup>40</sup> In this method, N methyl nicotinamide substances are first converted to trigonelline by alkaline hydrolysis. The "total" trigonelline is then determined and thus all methylated nicotinic acid derivatives known to be present in dog urine are accounted for.

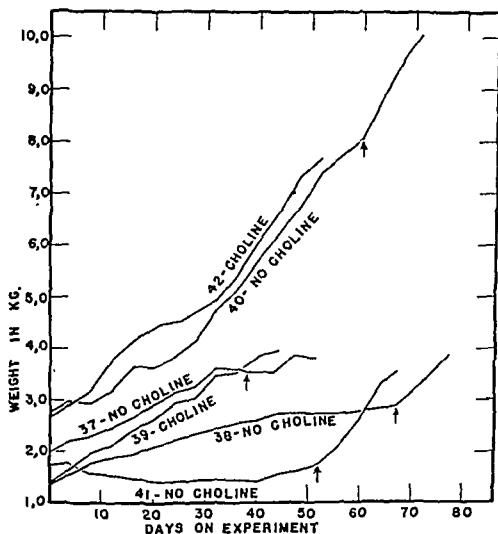


Fig. 1.—Growth curves of all the dogs used in these experiments. Arrows indicate beginning of period of choline supplement.

#### DISCUSSION

From Fig. 1 it can be seen that the two control dogs (Dogs 39 and 42) grew normally throughout the experimental period, although Dogs 37, 38, and 39 were a smaller, slower-growing breed than the others. The results of the liver function tests are given in Table I. It is seen that the control dogs gave normal results throughout the experimental period. Deficient Dogs 38 and 41 showed marked inhibition of growth and severe liver disease as judged by the liver function tests obtained on the sixty-eighth and fifty-third experimental days, respectively. Beginning on the sixty-eighth experimental day, 200 mg. per cent of choline chloride was added to the ration of Dog 38 and a total of 6.65 Gm. was given orally by gelatine capsule during the following ten days. This supplement produced striking improvement in liver function and all tests gave normal results by the tenth day. The dog was then sacrificed and the liver was found

<sup>40</sup>We are indebted to Dr. W. A. Perlzweig for generously furnishing us with trigonelline sulfate for use in this method.

TABLE I  
SUMMARY OF LIVER FUNCTION TESTS OF ALL DOGS

<i>Control Dog 39—200 mg. Per Cent Choline Chloride</i>					
Experimental day	18		24		39
Plasma phosphatase	79		170		144
Bromsulfalein test					6
<i>Control Dog 42—200 mg. Per Cent Choline Chloride</i>					
Experimental day	18		39		53
Plasma phosphatase	232		321		192
Plasma phosphatase in 0.05 M NaCN			2		
Plasma phosphatase in 0.01 M NaCN					0
Bromsulfalein test	10				8
Plasma total cholesterol	160				240
Plasma cholesterol esters	99				198
Prothrombin time					8.9
<i>Choline-Deficient Dog 37—Choline Started on Thirty-ninth Day</i>					
Experimental day	18		24		44
Plasma phosphatase	853		1340		246
Bromsulfalein test					10
Prothrombin time					12.4
<i>Choline-Deficient Dog 40—Choline Started on Sixty-first Day</i>					
Experimental day	18	32	39	53	61
Plasma phosphatase	1040	593	641	763	586
Plasma phosphatase in 0.05 M NaCN		4	4		
Plasma phosphatase in 0.01 M NaCN				200	170
Bromsulfalein test	31	23		26	29
Plasma total cholesterol	116	169		166	158
Plasma cholesterol esters	69	91		86	99
Prothrombin time				10.9	
<i>Choline-Deficient Dog 38—Choline Started on Sixty-eighth Day</i>					
Experimental day	18	58	68	71	74
Plasma phosphatase	247	1435	1348	1088	651
Bromsulfalein test		26	38	20	12
Plasma total cholesterol		72	52	95	119
Plasma cholesterol esters		39	34	45	83
Prothrombin time			22.0	13.4	9.3
<i>Choline Deficient Dog 41—Choline Started on Fifty-third Day</i>					
Experimental day		39	53	57	61
Plasma phosphatase		1164	1481	854	371
Plasma phosphatase in 0.05 M NaCN		0			
Plasma phosphatase in 0.01 M NaCN			425	342	90
Bromsulfalein test			27	9	7
Plasma total cholesterol			54	118	174
Plasma cholesterol esters			30	79	111
<i>Plasma Phosphatase</i> —Micrograms of inorganic phosphorus liberated per cubic centimeter of plasma in twenty-four hours. Normal range for control pups <sup>1</sup> : 80 to 350. <i>Bromsulfalein Test</i> —Micrograms of dye per cubic centimeter of plasma at eight minutes. Normal range: 3 to 10 <i>Plasma Total</i> cent. Normal range: 100 to 250. <i>Plasma Chole</i> or cent. Normal range: 70 to 200. <i>Prothrombin</i> ge: 8 to 13.					

to contain a normal amount of lipid material as determined by chloroform extraction (12.4 per cent of dry weight). This indicates a rapid removal of liver lipid since the liver would be expected to contain 40 to 50 per cent lipid (dry weight) before choline therapy, as judged from previous studies with this degree of impairment of liver function.

With Dog 41 the ration was supplemented with 200 mg. per cent of choline chloride beginning on the fifty-third experimental day and a total of 2.38 Gm. orally by gelatine capsule during the following five days. Rapid improvement in liver function resulted from this supplement and normal liver function was obtained by all methods of testing on the eighth day following the inception of choline therapy. Striking increases in food consumption and body weight were produced in both Dogs 38 and 41 by the choline supplements. In neither dog, however, did the addition of choline to the diet produce any significant



increases in the daily per kilogram output of creatinine or in the excretion of methylated nicotinic acid derivatives in terms of the per cent of the total nicotinic acid intake. Dog 38, however, did show a significant increase in the per kilogram excretion of creatine after five days of choline supplementation. This was not observed in Dog 41 and has not been observed in other dogs.

Dogs 37 and 40 showed slight early growth impairment on the deficient diet and subnormal liver function. Thus Dog 37 showed a moderately high plasma phosphatase value on the eighteenth day which rose to a very high value on the twenty-fourth day. However, growth ceased shortly after and perhaps accounted for the spontaneous improvement in liver function observed on the thirty-ninth day. At this time, the dog was given 0.5 Gm. of choline chloride orally and was placed on the control ration. The plasma phosphatase value was normal five days later.

Dog 40 showed a slight pause in growth between the fifteenth and twenty-fifth day and poor liver function as measured by plasma phosphatase and brom-sulfalein elimination on the eighteenth day. Plasma cholesterol fractions at this time were not abnormally low, however. This animal showed a striking spontaneous improvement in liver function and gain in weight after this time although, as in the case of Dog 37, liver function did not return to normal. Beginning on the sixty-first day, the ration of this dog was supplemented with 200 mg. per cent of choline chloride. Again liver function tests were found to be normal five days later. No change in the creatinine excretion of Dog 40 was produced by the choline supplement, while the creatine excretion actually dropped slightly. The excretion of N methyl nicotinic acid derivatives was extremely variable in the two deficient periods studied and was somewhat elevated after the addition of choline to the ration. This spontaneous remission in Dog 37 and particularly in Dog 40 is remarkable and in both instances occurred in the largest dogs of the litter. We have previously observed "resistance" to choline deficiency (Dog 29, Experiment 5).<sup>1</sup> This dog, the largest of a litter of seven, showed good growth on an intake of only 5 mg. of choline per 100 Gm. of ration. It is possible that the choline requirement may be lowered after a certain stage in growth or development has been reached. The healing of kidney lesions in young rats surviving choline deficiency is a remarkable phenomenon and may be analogous to this conjuncture in the dog.

Judging from the changes in liver function produced by choline therapy in choline-deficient pups, it would appear that in severely deficient dogs (38 and 41), a week or ten days were required to produce normal liver function, whereas with less severe deficiency (Dogs 37 and 40) five days of therapy were sufficient. It must be emphasized, however, that rather large quantities of choline were given and that the liver disease was of a more acute than chronic nature. These factors would operate to give us a more rapid remission than would reasonably be expected under other circumstances. The index of liver function which gives the slowest return to the normal is the phosphatase level.

The effect of cyanide on the plasma phosphatase activity in these animals is of interest in connection with the findings of Drill and co-workers.<sup>2</sup> They observed that sodium cyanide in concentrations of 0.0001 to 0.1 M markedly inhibited the high serum phosphatase activity of dogs with hepatic damage produced by poisoning with carbon tetrachloride, by cholecystectomy, or by bile fistula. These concentrations of cyanide had only a slight inhibitory effect on normal dogs. They postulated that the increase in serum alkaline phosphatase in liver damage is due to an increase in a component phosphatase normally pres-

ent in only small amounts. We thought it would be of interest to see if this relationship would hold in the liver damage of choline deficiency. The addition of 0.05 M sodium cyanide to the substrate produced virtually complete inhibition of plasma phosphatase activity in Dogs 40, 41, and 42. The addition of 0.01 M sodium cyanide produced a marked inhibition in the plasma phosphatase activity of Dogs 40 and 41 and complete inhibition in Dog. 42. In Dog 41 the plasma phosphatase activity in the presence of cyanide is compared with the normal uninhibited activity throughout the course of remission following choline therapy. It can be seen that much of the high phosphatase activity of the deficient state is inhibited by cyanide but that the residual uninhibited portion is greater than that present in normal pups. As the plasma phosphatase activity decreases during the period of restoration of liver function, the cyanide insensitive portion decreases to a point far below the normal phosphatase activity. This was also observed in Dog 40. These results do not indicate a high specificity for plasma phosphatase components to cyanide inhibition since the activity may be completely inhibited by sufficient concentration of cyanide (0.05 M). If, indeed, there is a specificity at lower concentrations (0.01 M), it would appear that the plasma of the normal pup contains only a small fraction of cyanide-insensitive phosphatase activity which is proportionally increased along with the cyanide-sensitive components in severe choline deficiency. This does not imply a fundamental discrepancy between our results and those of Drill and co-workers<sup>5</sup> since they have used adult dogs and different techniques for the production of liver disease.

In Table II are given the results of the excretion of creatinine, creatine, and methylated derivatives of nicotinic acid. The excretion of creatinine was found to be constant in all the dogs and averaged from 18.7 to 22.6 mg. per kilogram of body weight daily in the various periods of study. We observed no daily variation in the creatinine output. The output of creatine varies greatly between different dogs, and we found considerable daily variation in the same dog. In general, the litter including Dogs 37 through 39 averaged a higher output of creatine per kilogram than did the litter including Dogs 40 through 42. The creatine output would appear, from our studies, to bear no consistent relation to the choline intake. Glycocyamine fed orally or injected subcutaneously did not appreciably increase either the creatine or creatinine output of Dogs 37 or 39. It seems clear from these studies that creatinine and probably creatine excretion are independent of dietary choline, as was found to be the case with muscle creatine content in our earlier studies. If creatine and creatinine formation do indeed depend on the labile methyl supply for their formation from glycocyamine,<sup>3</sup> there must be a high priority on the available methyl supply for their formation. This high priority forces a rather uneconomical drain on the methyl reserves when the creatine excretion is high, as in the case of one severely deficient dog (Dog 38), and this drain continues in the face of a progressively deteriorating liver function. This would indicate a lack of adaptation on the part of the dog to a low methyl supply. However, it is also possible that synthesis of these substances in the dog may also take place from processes other than the methylation of glycocyamine by methionine or choline.

Our observations on the excretion of N methylated nicotinic acid derivatives are in general agreement with those of creatinine and creatine. The excretion of these substances expressed in terms of per cent of theoretical from the known nicotinic acid intake varies considerably from day to day and between dogs. Severely deficient dogs appear to excrete as much of these substances as they

TABLE II  
SUMMARY OF EXCRETION STUDIES OF CREATINE, CREATININE, AND N METHYL NICOTINIC ACID DERIVATIVES

Dog	LITTER 1				LITTER 2			
	37	38	39	40	41	42	Choline	Choline
Supplement	None	None	Choline	None	None	Choline	Choline	Choline
Metabolism study (Days)	26	9	10	10	10	15	15	14
Days of experiment (Inclusive)	13-38	59-67	68-77	11-29	43-52	53-67	53-67	11-24
Average daily creatinine excretion (Mg./Kg.)	19.7	22.4	22.6	20.1	18.7	19.9	19.9	20.1
Average daily creatine excretion (Mg./Kg.)	41.1	28.8	47.3	26.3	12.3	14.1	14.1	12.1
Total nicotinic acid intake for period (Mg.)	178	346	346	673	132	515	515	545
Total nicotinic acid output as methylated derivatives for period (Mg.)	115	242	242	283	91	257	257	465
Per cent conversion of nicotinic acid to methylated derivatives		64.6	69.9	42.0	69.0	49.9	49.9	83.3

do when given choline. This is all the more surprising in view of the fact that the dog can also excrete nicotinic acid in the form of nicotinuric acid. This would be the expected form during a condition of methyl deficiency since no methyl is needed in this synthesis.

These observations question the possibility of testing the methyl reserves of dogs by observations of creatine or creatinine excretion or the excretion of methylated nicotinic acid derivatives following doses of nicotinic acid. Whether or not other substances which depend on labile methyl from choline or methionine for their formation would be useful in this connection remains to be determined.

#### SUMMARY

1. Addition of choline to the diet of the choline-deficient pup results in rapid increase in food consumption and weight, improvement in liver function, and withdrawal of lipid from the liver. Under the conditions of our experiments, normal liver function has been restored in from five to ten days.

2. Excretion of creatinine, creatine, and N methyl nicotinic acid derivatives does not appear to be influenced by choline deficiency in the young pup.

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### AN ADRENAL CORTEX STIMULATING SUBSTANCE IN FEMALE HUMAN URINE

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THE presence of cortinlike substances in human urine has been demonstrated by Dorfman, Horwitt, and Fish.<sup>1</sup> These substances are capable of increasing the resistance of adrenalectomized rats to low environmental temperatures and of raising the mean liver glycogen concentration to five to ten times the normal amount.<sup>2</sup> It has been shown many times that there are substances in the urine and serum of pregnant women which act biologically in a manner similar to certain hormones of the anterior pituitary gland, but there are only a few reports that deal with hormones of this nature which might stimulate the adrenal cortex to elaborate the substances noted by the authors mentioned. Tepperman, Engle,

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and Long<sup>3</sup> refer to certain experiments in which de Boissezon<sup>4</sup> may have been successful in demonstrating such a material in the urine of pregnant women, although they state that this is not certain. More recently Golla and Reiss<sup>5</sup> have shown that pregnant mares' serum increases the adrenal weight of hypophysectomized rats and nine-day-old chicks; they postulate the presence in the pregnant mares' serum of a new corticotropic factor. No reference has been found which deals with a corticotropic factor in normal human urine.

The present experiments demonstrate the presence of a substance or substances in normal female human urine which, when injected into guinea pigs, produce an increase in mitotic activity in the adrenal cortex and in this respect acts similarly to certain substances elaborated by the anterior pituitary gland. The various types of urine used are shown in Table I. In most instances normal urine was obtained from women approximately five days before menstruation, although in a few instances it was obtained earlier in the sexual cycle. Pregnancy urine was collected from women three to three and one-half months pregnant. The urine was divided into two or three portions. The first portion was used as untreated fresh urine; to a second part, in some instances, thymol was added to act as a preservative; to a third portion formalin was added in sufficient quantity to bring the concentration of this substance in the urine to that shown in the various groups in Table I. Urine to which formalin had been added was then kept at a temperature of 39° C. and at pH 8 for seven days before injection into guinea pigs. The test animals consisted of seventy immature female guinea pigs ranging in weight between 170 and 210 grams which were injected intraperitoneally twice daily with 1.0, 1.25, or 2 c.c. of female human urine of the various types shown in Table I for periods of two and four days. Twenty noninjected immature female guinea pigs of a similar weight range served as controls. Mitotic counts in the adrenal cortex were carried out according to a method previously described<sup>6</sup> and were recorded as the average number of mitoses per section.

#### RESULTS

The results tabulated in Table I show three levels of mitotic activity in the adrenal cortex: (1) an average control level of 5.4 mitoses per section, (2) an average count in animals injected with untreated and with thymol- and formalin-treated pregnancy urine of 7.6 and 7.1 mitoses per section, respectively, and (3) an average of 12.6 mitoses per section in guinea pigs receiving untreated and formalin-treated normal female urine. The significance of the differences

TABLE I

THE EFFECT OF VARIOUS TYPES OF HUMAN FEMALE URINE ON MITOTIC ACTIVITY IN THE ADRENAL CORTEX OF IMMATURE FEMALE GUINEA PIGS

EXPERIMENTAL GROUP	NUMBER OF GUINEA PIGS	AVERAGE NUMBER OF MITOSES PER SECTION OF ADRENAL CORTEX	PROBABLE ERROR
1. Controls	20	5.4	±0.34
2. Normal female urine			
Untreated	10	12.3	±1.25
With 0.5 per cent formalin	5	13.1	±2.89
3. Pregnancy urine			
Untreated	16	8.2	±1.64
With thymol	12	6.8	±1.15
With 0.5 per cent formalin	12	6.2	±1.13
With 0.75 per cent formalin	9	7.9	±1.13
With 1.0 per cent formalin	6	7.8	±0.46

TABLE II  
SIGNIFICANCE OF DIFFERENCE BETWEEN VARIOUS GROUPS\*

EXPERIMENTAL GROUPS COMPARED	FACTOR OF STATISTICAL SIGNIFICANCE
1. Controls vs. normal female urine, untreated and with formalin	4.6
2. Controls vs. pregnancy urine untreated and with thymol	2.1
3. Controls vs. pregnancy urine with formalin	2.2
4. Normal female urine untreated and with formalin vs. pregnancy urine untreated and with thymol	10.2
5. Normal female urine untreated and with formalin vs. pregnancy urine with formalin	7.0
6. Pregnancy urine untreated and with thymol vs. pregnancy urine with formalin	1.7

\*The factor of statistical significance was calculated from the formula  $A_1 - A_2 / SE_{A_1} - SE_{A_2}$  in which  $A_1$  and  $A_2$  represent the averages of the groups compared and  $SE_{A_1}$  and  $SE_{A_2}$  the standard errors of the factor of less than 2 is of no significance; a factor between 2 and 3 almost certain significant difference; greater than chance, while one over 3 denotes an

in results in these three groups has been tested in two ways. First calculations of the probable errors in all subgroups show that if we take into account these factors in comparing the results of the subgroups of one level with those of another level of mitotic activity, there is no overlapping of results. In the second place, the three major groups and various subgroups have been compared as shown in Table II. From the latter calculations it may be concluded that the differences obtained between animals injected with normal female urine and animals injected with pregnancy urine of various types are almost certainly significant, while the differences between the various groups receiving different types of pregnancy urine and controls are probably significant. On the other hand, treatment of both normal and pregnancy urine for twenty-four hours with various concentrations of formalin or, in the case of pregnancy urine, with thymol failed to alter significantly the results in these respective groups.

#### DISCUSSION

From these results it may be concluded that there is a substance present in the urine of sexually active nonpregnant women capable of producing a hyperplasia of the adrenal cortex of the guinea pig, and it is furthermore notable that such a substance is also present in the urine of pregnant women, although it may be there in smaller quantity or in association with some inhibitory substance, since the response is less marked than after the injection of normal female urine.

It is not possible to determine the origin of this substance from these experiments alone, although certain possibilities may be considered. In the first place, it may be that we are dealing with a metabolic derivative of the corticotropic factor of the anterior pituitary gland excreted in the urine which would then elicit a response in the adrenal cortex which is somewhat less than that observed with implants of fresh anterior pituitary substance. The response to normal female urine is only about half as great as that which we have observed with fresh human anterior pituitary gland in some as yet unpublished experiments. A similar relationship has been observed numerous times with respect to the estrogens elaborated by the ovary; in this case also certain urinary metabolites of estrogenic substances act in a manner qualitatively similar to, but qualitatively weaker than, the parent estrogens found in the ovaries. However, the factor of dilution in the urine must be considered before this conclusion can be made with certainty. To test this factor, in a few experiments a concentrated powder of pregnancy urine prepared by the method of Loeb and Hay-

ward<sup>7</sup> was injected into immature female guinea pigs. The results were not significantly different from those obtained with either fresh or formalin-treated pregnancy urine.

A second possibility which might be considered is that of a response of the adrenal cortex to ovarian hormones elaborated as a result of stimulating substances present in the urine. We have observed in some, as yet unpublished, experiments that administration of corpus luteum hormone produces an increase in mitotic activity in the adrenal cortex of the guinea pig, but this reaction is usually most marked in the zona glomerulosa as contrasted with that of anterior pituitary substance which induces mitotic activity almost exclusively in the outer half of the zona fasciculata. In our present experiments most of the mitotic figures were found in the outer half of the fasciculata, although in some experiments, specifically those with fresh pregnancy urine, there were in some cases as many as 15 per cent of the mitoses in the zona glomerulosa. This may be taken to indicate that the actual difference between normal and pregnancy urine is even greater than the results shown in Table I indicate, and that in the case of pregnancy urine there is in addition to the stimulus exerted by ordinary urine a stimulus of a different kind which affects the average number of mitoses per section in the glomerulosa. As further evidence against the assumption that luteal hormone is the agent present in the urine we might cite the observations of Loeb and Hayward,<sup>7</sup> that the luteinizing effect which pregnancy urine produces in the guinea pig ovary is inhibited by formalin treatment and replaced by a connective tissue ingrowth into the ovarian follicles. Thus, if luteal hormone were the major factor in inducing these proliferative effects, it should be expected that untreated pregnancy urine would call forth the greatest mitotic response in the adrenal cortex, since it produces the most marked luteinizing effect, and that treatment with formalin would diminish this response. It is shown in Table I that this is not the case, no essential difference being noticeable between normal and formalin-treated urine.

It is, therefore, most likely that we are dealing with a derivative of anterior pituitary substance or with some substance which acts in a similar manner. That this substance is different from the pituitary-like substance elaborated in the placenta is shown by the fact that normal female urine produces a greater response than pregnancy urine. It appears that in contrast with the conclusions of Golla and Reiss,<sup>5</sup> we are dealing with a substance which is not peculiar to the state of pregnancy but which occurs also in the urine of nonpregnant females and perhaps also in that of males, although the urine of males has not yet been studied from this point of view. This conclusion would still hold even if it were determined that in a later stage of pregnancy the urine is able to produce a mitotic response in the adrenal cortex greater than that observed after the injection of normal female urine. In a later stage the ovarian hormones might exert an influence sufficiently great to overcome such assumed antagonistic substances in the urine of pregnancy, or it is also conceivable that these latter substances present during the first trimester of pregnancy might gradually disappear as pregnancy progresses.

Finally, it would be emphasized that the demonstration of pituitary-like effects of some body fluid or of its excretion product in the urine on the adrenal cortex does not necessarily indicate that we are dealing with a new substance which has a specific corticotropic effect; it is more likely that this effect is due either to circulating pituitary hormone or to some metabolic derivative of it.

## SUMMARY

The corticotropic effect of normal and pregnancy human female urine has been studied. It has been found that normal human female urine produces a greater increase in mitotic activity in the adrenal cortex of immature female guinea pigs than does pregnancy urine. Treatment of this urine with various concentrations of formalin failed to alter the results appreciably. The likelihood that this corticotropic effect of urine is due to a metabolite of anterior pituitary corticotropic hormone is discussed.

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THE DETERMINATION OF PROTHROMBIN TIME FOLLOWING THE  
ADMINISTRATION OF DICUMAROL, 3,3'-METHYLENEBIS  
(4-HYDROXYCOUMARIN), WITH SPECIAL  
REFERENCE TO THROMBOPLASTIN\*†

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WHEN the Quick prothrombin time test was first used as a measure of bleeding tendency, particularly in hepatic disease, all the information that was actually necessary was whether the prothrombin time was normal or prolonged. The exact degree of prolongation or the exact amount of prothrombin deficiency was not particularly important. In recent years, the prothrombin time test has become the indispensable guide to the administration of dicumarol, 3,3'-methylenebis (4-hydroxycoumarin), and it is important to know not only the normal prothrombin time, but also the degree of prothrombin deficiency which has developed as a result of the dicumarol. In order that prothrombin times may give this information, it is necessary for the laboratory to standardize in detail the technique of the prothrombin time test as performed.

When prothrombin times are determined on prothrombin-deficient plasmas, it becomes apparent that the results obtained depend to a great extent on the activity of the thromboplastin employed, that in order to obtain consistent results from day to day it is necessary to employ thromboplastins of similar activity, and that to interpret adequately a particularly prothrombin time one must have certain information regarding the activity of the thromboplastin employed.

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Clinical studies<sup>2, 3</sup> have shown that after the administration of dicumarol, thrombosis almost certainly will not occur if the prothrombin time is greater than twenty-seven seconds (thromboplastin "A")\* of this study, normal prothrombin time, seventeen to nineteen seconds). bleeding of any significant degree is very unlikely to occur among patients who have received dicumarol if the prothrombin time is less than sixty seconds (thromboplastin "A"). Arbitrarily a prothrombin time of thirty-five seconds (thromboplastin "A") was established by Allen, Barker, and Waugh<sup>2</sup> as a guide to the dosage of dicumarol as follows: On days when the prothrombin time was less than thirty-five seconds, a dose of the drug was given; on days when the prothrombin time was greater than thirty-five seconds, no drug was given. Obviously these rather critical points—twenty-seven seconds, thirty-five seconds, and sixty seconds—applied only to the test as performed and to the thromboplastic substance which was used (thromboplastin "A").

Meyer, Bingham, and Axelrod<sup>24</sup> expressed the opinion that prolongation of the prothrombin time with dicumarol to between twelve and one-half and nineteen seconds is reasonably safe. These workers used a thromboplastin of such potency that with an optimal solution of calcium chloride, the normal prothrombin time was 9.5 to 10.5 seconds.

Since the effect of thromboplastin on coagulation time (prothrombin time) is being considered, a brief review of the coagulation process may be in order.

*Factors in Coagulation.*—A number of factors are concerned in the coagulation of blood. These include prothrombin, thromboplastin, calcium, thrombin, and fibrinogen, as well as certain inhibitors, among which are plasma antithrombin, plasma antiprothrombin, and heparin. According to the most commonly accepted hypothesis, blood clotting may be regarded as a two-stage process:

1. Prothrombin + thromboplastin + calcium = thrombin
2. Thrombin + fibrinogen = fibrin

The exact mechanism of the first stage of clotting still remains a subject of controversy. Whether the conversion of prothrombin to thrombin results from an enzymic reaction whereby thromboplastin alone or in conjunction with calcium acts as a catalyst or whether there is an actual chemical combination of prothrombin with either thromboplastin or calcium or both is not known. It has been suggested that thromboplastin merely neutralizes antiprothrombin, thus allowing prothrombin to react with calcium, forming thrombin. Regardless of which, if any, of these hypotheses is correct, it is evident that thromboplastin greatly accelerates the speed of clotting.<sup>28</sup> Most workers have agreed that the presence of this factor in tissues and blood platelets is necessary to the conversion of prothrombin to thrombin under normal conditions. Aqueous solutions of tissue extracts,<sup>28</sup> suspensions of platelets,<sup>8, 13</sup> cephalin,<sup>8, 16</sup> snake venom,<sup>14</sup> and trypsin<sup>15, 16</sup> are known to accelerate clotting time.

The chemistry of thromboplastic substances and their effects is not fully known, though studies have been made. Eagle<sup>14</sup> has shown that the coagulative action of venom is probably due to a proteolytic enzyme. It is known that clotting activators from tissue cells may be thermolabile and soluble in water or thermostable and soluble in alcohol or ether. The phosphatide fraction from platelets has been found to contain a potent activator.<sup>8</sup> Both a protein and a phosphatide component of the thromboplastic protein from beef lung were re-

\*See under Preparation of Thromboplastin.

quired for maximal thromboplastic action. The protein component was inactive after removal of the phosphatides.<sup>10</sup> Both "lecithin" and "cephalin" fractions of a complex phosphatide mixture extracted from the thromboplastic protein from lungs showed considerable clotting activity.<sup>11</sup> The "cephalin" fraction of brain phosphatides, formerly thought to be a definite compound, has been shown to be a mixture of phosphatides.<sup>18</sup> Fractional ultracentrifugation of saline extracts of beef lung has led to the isolation of a high molecular lipoprotein with high thromboplastic activity.<sup>9</sup> Chargaff,<sup>7</sup> in 1944, reported on the thromboplastic activities of numerous phosphatide fractions obtained from pig heart, beef heart, and beef brain. Phosphatidyl serine from beef brain was found to be inactive. Brain cephalin itself (that is, ethanolamine phosphoryl diglyceride) showed some activity. The conclusion was reached that "the thromboplastically active lipids cannot yet be identified with any of the known phosphatides."

Thromboplastic substances, aqueous tissue extracts,<sup>12, 32, 37</sup> and snake venom<sup>19, 26, 27</sup> in particular are used in laboratory tests for prothrombin. The thromboplastin employed has an effect on the rate of conversion of prothrombin to thrombin.

*Methods for Prothrombin Determination.*—The rate at which thrombin is formed is affected by the concentration of prothrombin. As the means of recognizing this factor is its capacity to form thrombin, any method for determination must be an assay based on formation of thrombin.<sup>4</sup>

The two-stage method of Warner, Brinkhous, and Smith<sup>37</sup> for prothrombin determination utilizes the biphasic nature of the clotting reaction. In the prothrombin conversion stage, prothrombin is converted completely to thrombin with an optimal amount of calcium and an excess of thromboplastin. In the clotting stage, the amount of thrombin formed is measured by the time required for the clotting of a standard solution of fibrinogen.

The one-stage method of Quick, Stanley-Brown, and Bancroft,<sup>32</sup> or some modification thereof, has been more widely adopted as a clinical test for prothrombin than the two-stage method. The one-stage method consists in determination of the clotting time of oxalated plasma at 37.5° C. after addition of an excess of thromboplastin and a fixed amount of calcium. Thus the time represents a summation of the conversion time and the clotting time. Quick stated that with the addition of a fixed quantity of calcium and an excess of thromboplastin, prothrombin is the only variable and the clotting time of the oxalated plasma can be considered a direct measure of the prothrombin concentration of the blood.

Dam and Glavind,<sup>12</sup> instead of determining prothrombin time with a fixed concentration of thromboplastin, determined the concentration of a tissue extract that would clot heparinized plasma in three minutes.

*Factors Influencing the Prothrombin Determination.*—Stewart and Pohle,<sup>36</sup> investigating the effect of calcium on the plasma prothrombin time, observed that the minimal coagulation time could be assured only by determining the optimal amount of calcium necessary for recalcification in each instance. Quick<sup>39</sup> agreed with them that clotting time could be shortened by reducing the strength of the calcium chloride, but he did not believe that calcium should be made a variable in the test.

Electrolyte concentration, pH, and temperature are known to influence the rate of conversion of prothrombin, and these factors should be kept constant in so far as is possible.

Differences in "convertibility" of prothrombin have been observed by some workers. Whether this is due to species differences or to unrecognized factors which influence clotting is not known.<sup>4</sup> The presence of inhibitors also affects the conversion rate. "Assay methods for thromboplastin, inhibitors, and the 'convertibility factor' are still incompletely developed and a complete quantitative analysis of the clotting problem is not at present available."<sup>34</sup>

In stating the underlying principle of the prothrombin time test, the expression "excess thromboplastin" is generally used. This does not mean, however, that the presence of an excess makes up for any lack of potency of the particular thromboplastin employed. The prothrombin time obtained depends, in part, on the activity of the thromboplastic substance.

*Factors Influencing the Activity of Thromboplastin.*—The factor having the greatest influence on the activity of thromboplastin prepared from rabbit brain is whether or not the brain has been extracted with acetone. It will be noted that the thromboplastin "A" used routinely in this study has been made from dried rabbit brain without previous extraction with acetone. This follows the original method of Quick, Stanley-Brown, and Bancroft.<sup>32</sup> Many workers, however, prefer Quick's modified method<sup>29</sup> in which he extracted the brain with acetone previous to the drying process. With the latter method it was possible for Quick to prepare a more active thromboplastin (normal prothrombin time, twelve to thirteen seconds), whereas he had previously obtained a less active preparation (normal prothrombin time, twenty-two to twenty-five seconds\*).

Unfortunately, thromboplastins prepared according to the same general method do not always possess similar activity. This is true regardless of the time which has elapsed between the preparation of the dried brain and preparation of the thromboplastin. It would not be profitable to enumerate every possible reason why individual preparations of thromboplastin may vary in activity. It should be realized, however, that the fineness of the powdered brain, the length of time and the temperature at which the saline extract is incubated, the amount of mixing prior to, during, and after incubation, and the period of centrifugation each may have an effect on the potency of the thromboplastin. When the brain is extracted with acetone, the length of time and the manner in which the extraction is carried out have an effect on the potency of the final thromboplastin. This effect may be due to differences in the amounts of either inactive or inhibitory substances removed by the acetone. Regardless of precautions taken, one may occasionally obtain a thromboplastin so different in activity from other preparations used that it is advisable to discard it.

The amount of dried brain extracted in the actual preparation of the thromboplastin may have considerable effect on the potency of the final strength, though the literature records considerable range in the initial concentration of the thromboplastins as prepared. Quick<sup>28</sup> has correctly stated that when the thromboplastic activity of increasing concentrations of a tissue extract was tested, the clotting time reached a minimum which could not be appreciably shortened by additional thromboplastin. One can go further, however, and say that as the concentration of thromboplastin is increased beyond the amount giving a minimal clotting time, the prothrombin time can be prolonged. This fact is not always evident when determinations are made on normal plasmas. But when thromboplastins of varying concentrations are tested with prothrombin-deficient plasmas, it will be noted that within certain limits of a selected prothrombin time, the time can be shortened or prolonged. This prin-

\*Quick stated that thromboplastin prepared from rabbits obtained in a different locality would clot human plasma in sixteen to seventeen seconds.

ciple has a very practical application in determining prothrombin times on patients who are receiving dicumarol.

*Preparation of Thromboplastin.*—Thromboplastin "A" was prepared by a method similar to that previously described.<sup>23</sup> A rabbit was killed by injecting 15 c.c. of air into the lateral vein of the ear. The skull was quickly opened and the brain was removed, freed of meninges and blood, and then triturated with a spatula on a glass plate (7 by 10 inches [18 by 25 cm.]). It was then spread over the plate and allowed to dry in an incubator (37.5° C.) for approximately twenty-four hours. The dried brain was scraped off, care being taken to scrape in such a manner that it did not become too finely powdered. It was then stored at 5° C. in a tightly covered glass jar. It has not been found necessary to store the dried brain in ampules. As mentioned elsewhere, twenty-four dried brains were usually mixed together. The number of brains to be mixed together would naturally depend on the number of prothrombin time tests being performed by the individual laboratory.

The actual preparation of the thromboplastin was as follows: A selected amount of dried brain was added to a known volume of physiologic saline solution, the concentration depending on the activity of the material used.\* The brain and saline solution were thoroughly mixed with the aid of a stirring rod, incubated at 56° C. for fifteen minutes, quickly cooled, again thoroughly mixed, and then centrifuged at low speed for not more than two minutes. The supernatant liquid is the "thromboplastin" and can be used as long as its activity is maintained. Obviously, the foregoing directions will be followed somewhat differently by different persons in so far as details are concerned, and thromboplastins of different activities may be obtained. This probably accounts in part for the variation in normal prothrombin time reported by various workers using the same prothrombin time test. However, the important thing is that each technician be consistent in the preparation of successive thromboplastins.

*Selection of a "Standard" Thromboplastin "A".*—The following procedure was adopted more than two years ago as a means of procuring thromboplastins of consistent activity. Thromboplastins were prepared from mixtures of the dried brains of twenty-four rabbits. Each mixture was kept at 5° C. and used over a period of approximately six weeks to two months. Each time a new mixture was tested, thromboplastins were prepared using 0.6, 0.8, and 1.0 Gm. of dried brain in 10 c.c. of saline solution. These preparations were tested with plasmas giving prothrombin times in the thirty-, forty-, and fifty-second range. Results obtained with two such mixtures are illustrated in Table I. The thromboplastin most nearly checking the one in use was selected (Table II). Concentrations ranging from 0.4 to 1.0 Gm. per 10 c.c. were sometimes used and it was usually possible to prepare a thromboplastin giving a somewhat shorter or longer prothrombin time, as was necessary to check a "standard" thromboplastin. A concentration of 0.6 or 0.8 Gm. per 10 c.c. was most often selected as being satisfactory. While normal plasmas were tested as well, it saved time and material to test plasmas with prolonged prothrombin times first.

The importance of checking each thromboplastin is shown in Table III. Unfortunately, thromboplastins prepared in like manner did not always possess similar activity. In this study each fresh preparation of thromboplastin "A" was checked with a previously "standardized" thromboplastin. Thrombo-

\*See under Selection of a "Standard" Thromboplastin "A."

TABLE I

PROTHROMBIN TIME DETERMINATIONS TO SHOW COMPARISON OF THROMBOPLASTINS PREPARED WITH VARYING AMOUNTS OF DRIED BRAIN OF RABBIT

RABBIT BRAIN MIXTURE	THROMBOPLASTIN "A" (GRAMS IN 10 C.C. SALINE)	PROTHROMBIN TIME, (SECONDS)					
		NORMAL	PATIENTS				
			1	2	3	4	5
I	0.6	17	27	33	37	41	52
	0.8	17	29	36	41	47	59
	1.0	17	32	38	43	49	63
II	0.6	NORMAL*	PATIENTS				
			6	7	8	9	10
			18	18	26	38	47
II	0.8	18	18	26	40	48	56
	1.0	18	18	28	45	53	65

\*Different normal from that used with mixture I.

TABLE II

COMPARISON BETWEEN THROMBOPLASTINS PREPARED WITH VARYING AMOUNTS OF A NEW MIXTURE AND A PREVIOUSLY STANDARDIZED THROMBOPLASTIN

RABBIT BRAIN MIXTURE	THROMBOPLASTIN "A" (GRAMS IN 10 C.C. SALINE)	PROTHROMBIN TIME, (SECONDS)				
		NORMAL	PATIENTS			
			1	2	3	4
Old	0.7*	18	26	35	46	75
	0.6	18	26	35	45	75
New	0.8	18	28	38	54	87
	1.0	21	34	54	78	141

\*Standardized.

TABLE III

VARIATION BETWEEN TWO THROMBOPLASTINS PREPARED SIMULTANEOUSLY AND TWO STANDARDIZED THROMBOPLASTINS

RABBIT BRAIN MIXTURE	THROMBOPLASTIN "A" (GRAMS IN 10 C.C. SALINE)	DATE OF PREPARA- TION	PROTHROMBIN TIME (SECONDS)				
			NORMAL	PATIENTS			
				1	2	3	5
I	0.5*	11/3/43	18	24	30	35	55
	0.5	11/5/43	18	24	30	35	56
	0.5	11/5/43	20	30	36	45	92
II	0.6*	11/5/43	18	23	30	34	56

\*Standardized.

plastin was considered to be "standardized" if it gave a normal prothrombin time of from seventeen to nineteen seconds,\* if it gave prothrombin times that were comparable to those obtained with thromboplastins which had been previously used, and if, in addition, it would give a prothrombin time of approximately thirty-five seconds when tested with a 20 per cent dilution of a selected normal plasma. Fortunately there seems to be little change from day to day in thromboplastins prepared as outlined earlier in this paper. Nevertheless, fresh solutions for checking purposes were prepared daily or on alternate days so that at least two thromboplastins giving satisfactory results were available. Often preparations five or six days old checked with the fresh preparations. To say that a single acceptable thromboplastin made up one day should automatically become the standard for comparison the day following might be misleading in some instances. However, the judicious use of the method of checking thromboplastins against each other as outlined has proved to be satisfactory, even though time consuming.

\*"Standardized" thromboplastins should give the same prothrombin times with the same normal plasma.

†Not more than 5 per cent variation.

Sometimes it was necessary to reduce the amount of dried brain weighed out in order to obtain a thromboplastin of the desired activity (Table IV).

Thromboplastins of the same concentration (0.8 Gm. in 10 c.c. of saline solution) prepared from different mixtures of dried rabbit brain sometimes showed considerable variation in their activities as evidenced by results obtained on prothrombin-deficient plasmas (Table V). When one thromboplastin was prepared which gave a shorter and another which gave a longer prothrombin time than a "standardized" thromboplastin, a suitable preparation was often obtained by mixing the two in proper proportions (Table VI). This procedure often saved time and material.

TABLE IV

VARIATION BETWEEN TWO DIFFERENT AMOUNTS OF SAME MIXTURE OF RABBIT BRAIN (MIXTURE I) AND PREVIOUSLY STANDARDIZED THROMBOPLASTINS

THROMBOPLASTIN "A" (GRAMS IN 10 C.C. SALINE)	DATE OF PREPARA- TION	PROTHROMBIN TIME (SECONDS)				
		NORMAL	PATIENTS			
			1	2	3	4
0.6*	10/22/43	18	26*	31	36	44
0.6*	10/22/43	18	27	30	36	45
0.6	10/25/43	18	29	35	41	52
0.5	10/25/43	18	26	30	35	44

\*Previously standardized.

TABLE V

COMPARISON OF THROMBOPLASTINS PREPARED SIMULTANEOUSLY FROM FOUR DIFFERENT MIXTURES OF DRIED RABBIT BRAIN\*

RABBIT BRAIN MIXTURE	PROTHROMBIN TIME (SECONDS)				
	NORMAL	PATIENTS			
		1	2	3	4
I	18	20	31	42	76
II	18	22	35	44	83
III	19	22	34	46	83
IV	19	23	37	53	102

\*Thromboplastin "A," 0.8 Gm. in 10 c.c. of saline solution, was used.

TABLE VI

EFFECT OF MIXING A MORE ACTIVE WITH A LESS ACTIVE PREPARATION OF THROMBOPLASTIN

THROMBOPLASTIN "A"*	PROTHROMBIN TIME (SECONDS)					
	PREPARATION	NORMAL	PATIENTS			
			1	2	3	4
I†	18	25	36	54	59	
II	18	22	31	44	49	
III	18	27	41	63	72	
1 part II	18	23	33	49	54	
1 part III						
1 part II						
2 parts III	18	25	35	55	60	

\*0.6 Gm. in 10 c.c. of saline solution.

†Standardized.

As a plan for testing any thromboplastin of unknown activity it is recommended that thromboplastins be "standardized" to the extent that successively used thromboplastins give the same prothrombin times on the same normal plasmas and, in addition, give comparable prothrombin times on prothrombin-deficient plasmas or give consistent results when tested with a 20 per cent dilution of selected normal plasmas.

*Technique of Prothrombin Time Test.*—The Quick prothrombin time test, as described by Magath,<sup>23</sup> was used. Whole blood was added to tenth-molar solution of sodium oxalate in the proportion of 9:1. After it had been mixed thoroughly, the blood was allowed to sediment or was centrifuged for a short period at low speed. One-tenth cubic centimeter of plasma was placed in a tube (13 by 100 mm.) and 0.1 c.c. of thromboplastin added to the plasma. The tube with contents was held in a constant temperature bath (37.5° C.). After a few moments, 0.1 c.c. of fortieth-molar solution of calcium chloride anhydrous was added and at the same instant, a stopclock (an electric clock operated by a foot switch has been used to good advantage) was started. The tube was carefully agitated and then tilted at intervals to determine the moment at which a semisolid clot was formed. With thromboplastin "A," the normal prothrombin time was found to be from seventeen to nineteen seconds.

*Correlation of Prothrombin Times Obtained With Thromboplastins of Different Activity.*—To interpret prothrombin times obtained with a less active thromboplastin in terms of those obtained with a more active thromboplastin presents a problem. In view of this fact it was decided to give some attention to thromboplastins made from acetone-extracted brain. Prothrombin time determinations were performed with thromboplastins (thromboplastin "B") made with varying amounts of the same mixture and with the same amounts of different mixtures of acetone-extracted brain (Tables VII and VIII).

TABLE VII

COMPARISON OF THROMBOPLASTINS PREPARED WITH VARYING AMOUNTS OF THE SAME MIXTURE OF ACETONE-EXTRACTED BRAIN

THROMBOPLASTIN "B" (GRAMS IN 5 C.C. SALINE)	PROTHROMBIN TIME (SECONDS)				
	NORMAL	PATIENTS			
		1	2	3	4
0.2	12	12	17	18	27
0.3	12	12	19	19	30
0.4	13	13	22	22	42
0.5	13	14	24	24	55

TABLE VIII

COMPARISON OF THROMBOPLASTINS PREPARED SIMULTANEOUSLY FROM THE SAME AMOUNT OF FOUR DIFFERENT MIXTURES OF ACETONE-EXTRACTED BRAIN\*

RABBIT BRAIN MIXTURE	PROTHROMBIN TIME (SECONDS)				
	NORMAL	PATIENTS			
		1	2	3	4
I	12	17	20	24	27
II	12	18	21	26	30
III	13	20	23	30	39
IV	15	23	27	35	46

\*Thromboplastin "B," 0.2 Gm. in 5 c.c. of saline solution, was used.

Prothrombin times were then determined on 100 subjects with three different thromboplastins: thromboplastin "A," average normal, eighteen seconds (prepared according to original method); thromboplastin "B,"\* average normal, twelve seconds (prepared according to Quick's modification); thromboplastin "D," average normal, twelve seconds (a commercial preparation†). The correlation between "A" and "B" is shown in Fig. 1; that

\*Preparations from selected mixtures of acetone-extracted brain were used; thromboplastins from different mixtures did not always possess similar activity.

†Bacto-thromboplastin supplied through the courtesy of Difco Laboratories, Inc., Detroit, Mich. Thromboplastins of the same lot number apparently possessed similar activity. Some variation existed between thromboplastins from different lot numbers. Further study would have to be made to decide definitely if the variation is significant.

between "A" and "D" in Fig. 2. The prothrombin time which one might expect with thromboplastin "D" if a particular prothrombin time is known for thromboplastin "A" can be obtained from the equation  $Y = 2.80 + 0.55X$  (Fig. 2). For example, if a prothrombin time of forty-five seconds has been obtained with thromboplastin "A," one would probably obtain a prothrombin time close to twenty-seven seconds if thromboplastin "D" were used. This would hold true of course only if preparations of standard activity were used.

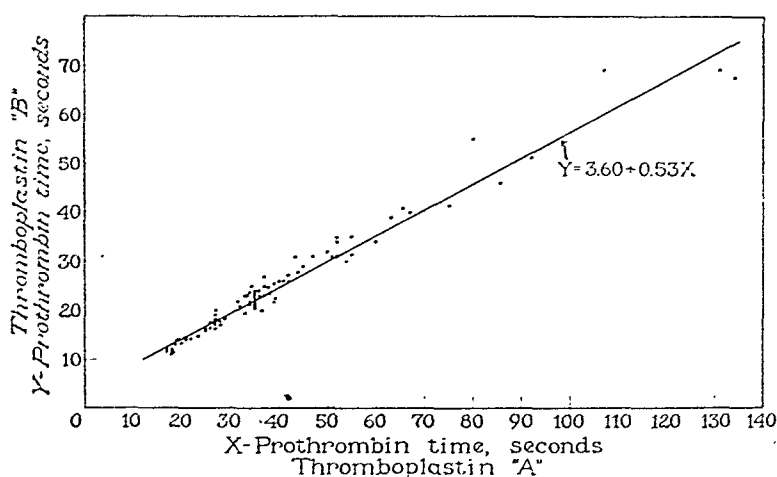


Fig. 1.—Correlation between prothrombin times obtained with thromboplastin "A" and thromboplastin "B."

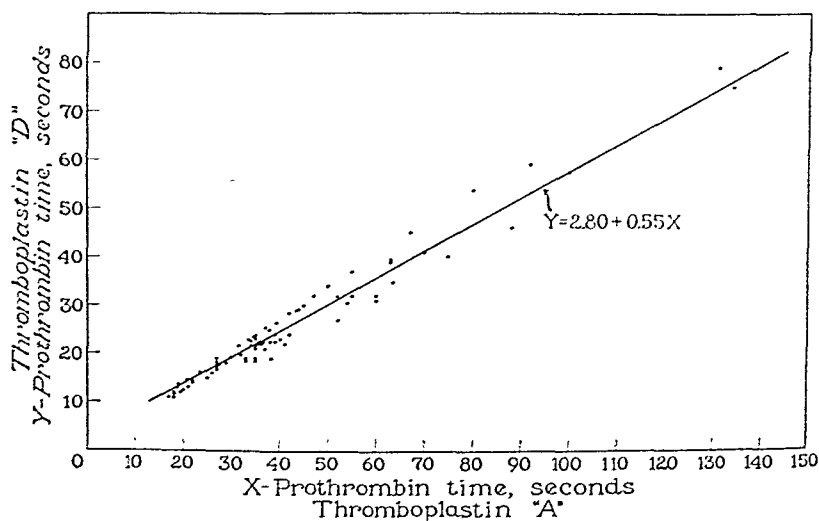


Fig. 2.—Correlation between prothrombin times obtained with thromboplastin "A" and thromboplastin "D."

In addition to this study, prothrombin times with the three different thromboplastins were determined on six patients who were receiving dicumarol. The results with two of the thromboplastins are shown in Fig. 3. Thromboplastin "B" gave results so close to those from thromboplastin "D" that the corresponding curve was omitted.

**Prothrombin Times and Prothrombin Percentage.**—In an effort to determine the significance of certain prothrombin times in terms of percentage of

\*The equation was obtained by the method of least squares by Mr. Robert Gage, of the Division of Biometry and Medical Statistics of the Mayo Clinic.



normal prothrombin, prothrombin time tests were performed on a series of dilutions of ten normal plasmas using thromboplastin "A" and thromboplastin "D." Results are shown in Table IX; an average curve for each thromboplastin is shown in Fig. 4. One cannot say how much prothrombin

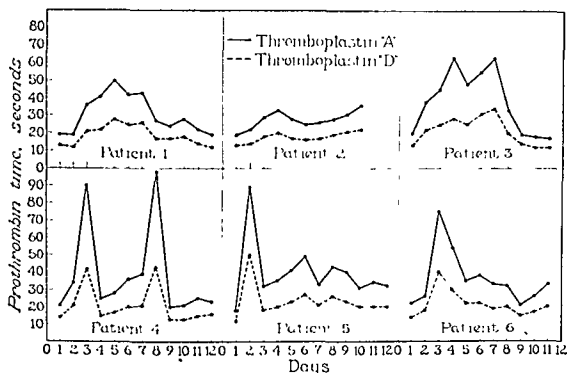


Fig. 3.—Comparison of prothrombin times obtained with thromboplastin "A" and thromboplastin "D" on six patients who were receiving dicumarol

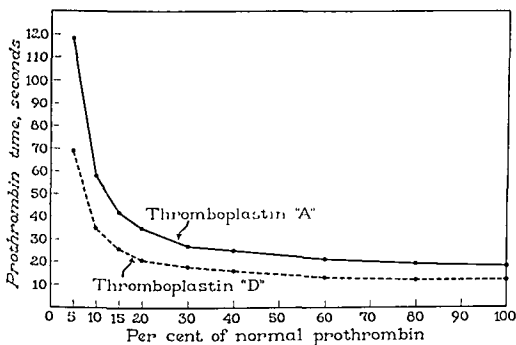


Fig. 4.—Prothrombin dilution curves. Relation between prothrombin time and percentage concentration of normal plasma expressed as per cent of normal prothrombin.

TABLE IX

AVERAGE PROTHROMBIN TIMES ON A SERIES OF DILUTIONS OF TEN NORMAL PLASMAS USING THROMBOPLASTINS "A" AND "D"

PER CENT NORMAL PLASMA	PROTHROMBIN TIME (SECONDS)	
	THROMBOPLASTIN "A"	THROMBOPLASTIN "D"
100	18	12
80	19	12
60	21	13
40	25	16
30	27	18
20	35	21
15	43	26
10	58	35
5	118	69

is present in normal plasma, but for purposes of comparison the original plasma without further dilution was considered to have 100 per cent prothrombin and each normal plasma was so diluted that it was considered equivalent to 80, 60, 40, 30, 20, 15, 10, and 5 per cent of normal prothrombin. Dilutions were made with prothrombin-free plasma, prepared according to the Seitz method of Kark and Lozner.<sup>21</sup> When physiologic saline solution was used as a diluent, end points were difficult to determine when the concentration of plasma was less than 20 per cent. Prothrombin times determined on diluted plasmas, when concentrations were 20 per cent or more, were essentially the same with either diluent when thromboplastin "A" was used. With thromboplastin "D," prothrombin times on diluted plasmas were somewhat prolonged when saline solution was used. Some investigators<sup>20, 36</sup> have recommended the use of fibrinogen solution as a diluent in the preparation of diluted plasmas.

When one applies prothrombin times from the appropriate curve in Fig. 4 to the equation,  $Y = 2.80 + 0.55X$  (Fig. 2), it will be seen that the calculated prothrombin times for the various dilutions closely check the observed prothrombin times for the same dilutions. Theoretically and by actual experiment, twenty-seven, thirty-five, and sixty seconds (actually fifty-eight seconds) obtained with thromboplastin "A" become equivalent to eighteen, twenty-one, and thirty-five seconds with thromboplastin "D." Again, this correlation holds true only when selected or "standardized" thromboplastins are used. Though one hesitates to say that when a particular prothrombin time is equivalent to that obtained with a 20 per cent dilution of normal plasma, the plasma being tested therefore contains 20 per cent of prothrombin, yet for purposes of comparison, dilution experiments provide certain information. Dilution may have an effect on certain coagulation factors. It is thought, for example, that dilution reduces the effect of clotting inhibitors.

*Critical Levels of Prothrombin.*—Prothrombin times of twenty-seven seconds and sixty seconds indicate critical levels of prothrombin when thromboplastin "A" has been used in the prothrombin time test. A prothrombin time of thirty-five seconds (thromboplastin "A") has been used as a guide to dosage of dicumarol. Because of the difficulty of comparing these prothrombin times with those considered significant when a thromboplastin of different activity has been used, it has seemed advisable to express twenty-seven, thirty-five, and sixty seconds in percentage of normal prothrombin. On examination of the appropriate curve in Fig. 4, it will be noted that, when thromboplastin "A" is used, these prothrombin times of twenty-seven, thirty-five, and sixty seconds correspond to the prothrombin times obtained with 30, 20, and 10 per cent normal plasma, respectively. Prothrombin times of eighteen, twenty-one, and thirty-five seconds were obtained on these same dilutions when thromboplastin "D" was employed. Since prothrombin times of twenty-seven, thirty-five, and sixty seconds (thromboplastin "A") corresponded to eighteen, twenty-one, and thirty-five seconds (thromboplastin "D") when original plasmas were tested (Fig. 2), it seems to justify further this method of comparing prothrombin times obtained with two different thromboplastins.

If one is using a thromboplastic substance of different or unknown potency and one wishes to select a prothrombin time equivalent to the thirty-five seconds obtained with thromboplastin "A," the most satisfactory method would be to determine the prothrombin times on 20 per cent dilutions of ten normal plasmas. The average of these determinations would give a prothrombin time which could serve as a guide to dicumarol dosage as outlined by Allen, Barker,

and Waugh.<sup>2</sup> Prothrombin times indicative of 30 and 10 per cent levels of normal prothrombin could be ascertained by determining the prothrombin times on the corresponding dilutions. Comparison of prothrombin times obtained with a 20 per cent dilution of normal plasma with those obtained with a prothrombin-deficient plasma containing approximately 20 per cent prothrombin is shown in Tables X and XI. Thromboplastins of different activity were employed.

TABLE X

COMPARISON OF PROTHROMBIN TIMES ON A 20 PER CENT DILUTION OF NORMAL PLASMA WITH THOSE OBTAINED ON A PROTHROMBIN-DEFICIENT PLASMA USING THROMBOPLASTIN "A"

PLASMA		PROTHROMBIN TIME (SECONDS)				
		I*	II	III	IV	V
Normal	Original	18	18	18	18	18
	20 per cent dilution	35	38	41	41	43
Prothrombin-deficient†	Original	34	36	38	39	40

\*Preparations of thromboplastin "A" of different activities.

†Patient receiving dicumarol.

TABLE XI

COMPARISON OF PROTHROMBIN TIMES ON A 20 PER CENT DILUTION OF NORMAL PLASMA WITH THOSE OBTAINED ON A PROTHROMBIN-DEFICIENT PLASMA USING THROMBOPLASTIN "B"

PLASMA		PROTHROMBIN TIME (SECONDS)				
		I*	II	III	IV	V
Normal	Original	12	13	15	16	17
	20 per cent dilution	22	26	30	35	35
Prothrombin-deficient†	Original	21	25	29	35	36

\*Preparations of thromboplastin "B" of different activities.

†Patient receiving dicumarol.

*Dilution of Normal Plasma as a Means of Evaluating the Activity of Thromboplastin.*—A dilution curve of normal plasma has been used by numerous workers to determine the percentage of prothrombin present in the blood. Since the curve obtained depends in part on the thromboplastin employed, it may well be used as a method of evaluating the activity of a particular thromboplastic substance. As a routine procedure the establishment of two or three selected points on the curve would probably give the necessary information. Dilutions should be made from a selected normal plasma (one previously studied) or from a sufficient number of normal plasmas to obtain what seems to be a representative curve. When the activities of different preparations of thromboplastin, prepared simultaneously, are being compared, the method of choice is to use dilutions of the same plasma. Some apparently normal plasmas may give curves at somewhat different levels. For this reason the use of selected plasmas will minimize the influence of other coagulation factors on the coagulation time obtained.

## COMMENT

Some comment is probably in order concerning the continued use of Quick's original method. When the modification was first proposed by Quick, it was realized that for the clinician to interpret results which would be obtained in terms of those with which he was already familiar would involve performance of both methods for some time. Furthermore, the possible advantages did not seem to outweigh certain disadvantages, the chief one being that such an active preparation could hardly show actual differences in prothrombin content that

would be apparent with a less active preparation. Souter and Kark,<sup>35</sup> in 1940, made a similar observation and stated that "in a test such as this when the time of the reaction represents the concentration of one interacting substance, it is essential for accurate estimation of the limits of that substance present that the decrease in its concentration should be reflected by an appreciable prolongation of the time of the reaction."

With the advent of dicumarol therapy, the problem was reconsidered. In addition to the conclusion drawn previously, which seemed to apply now more than ever, it was felt that the use of acetone-extracted brain did not assure more consistent results than when brain not extracted with acetone was used. True, the number of seconds of variation was usually less, but when one considered the change in prothrombin percentage or activity presented by the difference in time, interpretation of the results became more difficult. Thromboplastins prepared from different mixtures of acetone-extracted brain showed greater variation in their activities than did those prepared from the same mixture. This was partly due to the manner in which the brain was extracted with acetone. Standardization of details in the method of extraction eliminated marked differences in the normal prothrombin times but did not eliminate significant differences when prothrombin times were prolonged.

Quick<sup>31</sup> has stated that, because of the explicit directions which he has given for the preparation of a highly active thromboplastin, there is no longer valid excuse for reporting prothrombin data based on thromboplastin which does not give a normal prothrombin time of twelve and one-half seconds. If one assumes that this be true, the fact still remains that the use of such a thromboplastin would not necessarily assure consistent results on prothrombin-deficient\* plasmas. This, after all, is the real test of thromboplastic activity. It may seem that greater variation than 5 per cent should be allowed in prothrombin times obtained with prothrombin-deficient plasmas. But since thromboplastins are frequently prepared which check almost to the second when the prothrombin percentage is between 5 and 10 per cent, one cannot help feeling that when it is more than 10 per cent, differences in prothrombin time should represent differences in prothrombin and not differences in thromboplastic activity. Perhaps one is not justified in spending so much time to obtain consistent results with a test which is dependent on so many variables. But because prothrombin times thus obtained have been of great value from a clinical standpoint, one hesitates to depart from this established system of "standardization." Some state that they do not find variations in thromboplastins as prepared by them. However, since others do find such variation, the problem cannot be ignored.

When one considers the complexity of the factor or factors possessing thromboplastic activity, which are present in tissue, it is hardly possible that all preparations of any tissue extract should be equally potent. The presence of clotting inhibitors in tissue has been demonstrated.<sup>6</sup> Clotting inhibitors as well as clotting activators may be responsible for some of the variation which occurs in the activity of tissue extracts.

Souter and Kark<sup>35</sup> have recommended the preparation of stable thromboplastin by utilizing the method of "lyophilization" introduced by Flosdorf and Mudd.<sup>17</sup> This is a complicated procedure not easily adapted to a clinical laboratory. While it would have certain advantages over other procedures if it could be simplified, it would seem that some standardization as to activity should be made before the thromboplastin is lyophilized.

\*In this study, the expression "prothrombin-deficient" is used to designate those plasmas giving prolonged prothrombin times.

As has been shown, reducing the concentration of a tissue extract, under certain conditions, increases its activity. A similar observation was made by Aggeler and Lucia<sup>1</sup> and by Kazal and Arnow,<sup>22</sup> who noted that dilution of a thromboplastin increased its activity. It is possible that making serial dilutions of a particular thromboplastin might enable one to select a preparation of the desired activity.

Though prothrombin time tests using Russell viper venom have been performed in this study, comment concerning the results will not be made at this time. Numerous workers<sup>19, 26, 37, 39, 40</sup> have substituted venom for tissue extract in the Quick prothrombin time test. Worthy of mention is the difference in prothrombin time obtained with venom alone and venom plus lecithin. Witts and Hobson<sup>39</sup> have termed this the "lecithin difference." Witts<sup>38</sup> has referred to the influence of platelets on the effect of Russell viper venom, an influence which was comparable to that of lecithin.

Some investigators have followed the modification by Link and his associates<sup>5</sup> of Quick's test by determining the prothrombin time on diluted plasma in order to measure the effect of dicumarol. This has not seemed necessary as a routine procedure, at least when the less active thromboplastin (thromboplastin "A") was used. Whether the use of a diluted plasma in addition to undiluted plasma does furnish more accurate information in specific instances, as to the amount of prothrombin contained in the plasma,<sup>33</sup> is being studied further.

Since plasma dilution curves may be influenced by variables, known and unknown, it is well to keep in mind that a plasma giving a prothrombin time equivalent to that obtained with a 20 per cent dilution of normal plasma may not actually contain 20 per cent of normal prothrombin. However, the dilution curve method would seem to be the best method at our disposal to obtain an expression of prothrombin times in terms of prothrombin percentages. If the two-stage method of determining prothrombin were more applicable to clinical problems, the simultaneous performance of the two methods might furnish valuable information.

In particular, the dilution curve of normal plasma gives important information as to the activity of the thromboplastin employed. This is true, not only when comparing the activity of one thromboplastin with that of another,\* but especially when establishing critical levels of prothrombin deficiency. Since prothrombin time with any given method is determined not only by prothrombin content, but also by thromboplastic activity, the dilution curve best depicts the influence of these two coagulation factors. When thromboplastin "A" was used, it has been shown that the critical prothrombin times of twenty-seven, thirty-five, and sixty seconds might preferably be expressed as 30, 20, and 10 per cent of normal prothrombin. For purposes of comparison, prothrombin percentages† are more easily evaluated than are prothrombin times in seconds. It is absolutely essential, however, that each investigator establish a normal dilution curve for the method as performed. Prothrombin times should not be expressed as prothrombin percentage on the basis of a dilution curve obtained by someone else. In some instances dilution curves obtained by different investigators may be the same, but this is not necessarily so.

\*Nygaard<sup>28</sup> found that the correlation of prothrombin time in seconds and varying concentrations of a thromboplastin emulsion presented a better check as to potency than could be obtained by a single observation.

†Some ambiguity of expression exists when the ratio of a particular prothrombin time to the normal prothrombin time is expressed as per cent of normal or per cent prothrombin. Thirty-five seconds may denote a clotting activity equal to 51 per cent of that indicated by a normal time of eighteen seconds, but it is equivalent only to 20 per cent of normal prothrombin concentration. The relationship between prothrombin time and prothrombin concentration is not linear.

Hoffman and Custer<sup>20</sup> stated that "thromboplastin of optimal potency has probably not yet been obtained and many reports have appeared indicating that the standard and near optimal potency described by Quick was not used. When thromboplastin becomes a limiting factor, absolute values are no longer comparable and relative ones (i. e., per cent of normal) have not been shown to be strictly so."

In this study considerable emphasis has been placed on the factors, thromboplastin in particular, which may influence the prothrombin times obtained with the Quick prothrombin time test. However, this in no way is meant to minimize the importance of the Quick prothrombin time test, a test which continues to be the indispensable guide to dicumarol therapy.

#### SUMMARY

The Quick prothrombin time test is a reliable guide to the administration of dicumarol, provided thromboplastins of constant potency are used.

Prothrombin time tests have been performed simultaneously using a less active thromboplastin (thromboplastin "A," average normal, eighteen seconds) and more active thromboplastins (thromboplastins "B" and "D," average normal, twelve seconds).

Prothrombin times, indicative of critical levels of prothrombin, expressed as prothrombin percentages enable one to compare results obtained with thromboplastins of different activities.

The important levels of prothrombin deficiency during dicumarol therapy are: the level below which thrombosis is unlikely to occur, the level which serves as a guide to dicumarol dosage, and the level above which bleeding is unlikely to occur.

With thromboplastin "A" these levels have been established as 30 per cent (twenty-seven seconds), 20 per cent (thirty-five seconds), and 10 per cent (sixty seconds), respectively.

With thromboplastins "B" and "D" the prothrombin times denoting these critical levels have been established as eighteen seconds (30 per cent), twenty-one seconds (20 per cent), and thirty-five seconds (10 per cent).

Prothrombin times denoting critical levels when still other thromboplastins are used may be ascertained by determining the prothrombin times on appropriately diluted normal plasmas.

The activity of a particular thromboplastin may be evaluated by determining the prothrombin times on a series of dilutions of normal plasma.

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The coronary ostia were wide and the coronary arteries of normal caliber throughout with smooth glistening intima. The ascending aorta was of uniform diameter with thin, resilient walls and smooth intimal surface. There were a few minimal atheromatous deposits in the intima. There was a normal configuration of the major vessels arising from the arch.

*Lungs:* The left lung weighed 300 grams and the right 350 grams. Both lungs were uniformly fairly well collapsed. The external surfaces of all lobes were grayish-pink except for a well-outlined portion of both lower lobes which was dark blue. The visceral pleura, especially on the interlobar surfaces, showed fairly numerous petechiae. Sections through both lower lobes showed a deep red nonrepitant area which was sharply demarcated from the normal-appearing parenchyma. There were no focal lesions and no increased fluid present in this collapsed area. The bronchi of both lungs were largely filled with bloody mucopurulent material but contained no actual clotted blood.

*Spleen:* The spleen weighed 400 grams. It showed approximately normal contour with some notching of the anterior border. The external surface was reddish-blue in color with a thin, smooth capsule. The consistency was moderately soft. Section showed a dark red friable parenchyma in which a few small follicles were visible.

*Kidneys:* The left kidney weighed 250 grams and the right 250 grams. Both were of normal size and shape and were firm in consistency, the capsule strips readily leaving a smooth, reddish-brown surface. Section showed a normal configuration of medulla and cortex with prominent cortical striations. The right renal pelvis contained clotted blood. Both ureters were normal in caliber and position and contained no clotted blood.

*Liver:* The liver weighed 2,900 grams. It was moderately enlarged and showed smooth rounded contours and normal lobar configuration. The consistency was moderately firm and the color reddish-brown. The lobular markings were distinct.

The pertinent microscopic findings were as follows:

*Tumor:* Numerous sections from various areas of the mediastinal tumor showed a varied appearance. There was very extensive necrosis occupying two-thirds of the total tumor tissue. In the better-preserved areas there was irregular arrangement of varied tissues including glands of various types, nests of squamous epithelium, myxomatous tissue, and cartilage. In some areas the appearance was that of a highly malignant tumor but there was marked variation in the picture from one area to another. In other areas the picture was that of fibrosarcoma or reticulum cell sarcoma. In still other areas there were definite nests of anaplastic epithelial cells and glands, while still other areas showed cells of two different types, large and small, in irregular arrangement, suggesting seminoma. Occasional areas showed infiltration by small round and plasma cells.

*Mediastinal Lymph Nodes:* In one section the subcapsular sinuses were filled with large tumor cells. These occurred as individual cells and the lymph node structure was not disturbed. Another section showed widespread infiltration of the sinuses by similar tumor cells but again the lymph node structure was not destroyed.

*Bone Marrow:* Sections from sternum and ribs showed almost complete replacement of normal marrow by anaplastic tumor tissue containing numerous giant cells and many mitotic figures. There was very extensive necrosis of the tumor and marrow with fairly marked destruction and fibrous tissue replacement of the marrow cells.

*Spleen:* The spleen was very cellular with prominent follicles. Anaplastic tumor cells were present throughout the pulp and sinuses, both diffusely and in groups. Many of them were giant cell forms with several nuclei, while mitotic figures and partially necrotic cells were plentiful. There were immature myeloid cells, a number of them eosinophilic myelocytes.

*Liver:* The liver cells had a foamy appearance due to glycogen. The sinusoids contained numerous immature cells, many showing mitotic figures. Some of these resembled blast forms of the myeloid series; others were apparently tumor cells. There was no leucemic infiltration or areas of hemopoiesis.

The final anatomical diagnoses were as follows:

1. Perforating needle wound of sternum and pericardial sac.
2. Laceration of anterior wall of right ventricle with hemopericardium and cardiac tamponade.
3. Malignant teratoma of anterior mediastinum with metastases to mediastinal lymph nodes, bone marrow of sternum and ribs, spleen, and sinusoids of liver.
4. Splenomegaly with myeloid metaplasia.
5. Hepatomegaly.
6. Petechial hemorrhages of skin and massive hemorrhage into renal pelvis.



7. Partial atelectasis of both lower pulmonary lobes.
8. Superficial ulceration of left small toe.

### DISCUSSION

Meyer and Halpein\* reported the first death from sternal puncture. As in our case, they experienced difficulty in aspirating marrow, and their patient died shortly thereafter with feeble, rapid pulse, unobtainable blood pressure, and distended neck veins. Their patient experienced no pain other than that which might be expected in an individual made more apprehensive by unsuccessful attempts at aspiration. The same may be said of our patient. Unable to secure autopsy, Meyer and Halpein attributed death to "cardiac inhibitory reflexes through the vagus, initiated by fear." As the signs in the two cases were identical, it seems not unlikely that their patient also died of cardiac puncture and tamponade. Cardiac tamponade is accompanied by pallor with distended neck veins, while on the other hand vasovagal syncope (shock) is accompanied by pallor but with completely collapsed veins.

In a large series extending back to 1936, one of us (J. H. S.) has experienced no other accident or sequela of any type in performing sternal puncture. With the exception of the first year or two, he has used an 18-gauge spinal needle sawed off and rebevelled to a length of about  $1\frac{1}{4}$  inches and has employed the technique of boring directly down into the mid-sternum at the level of the third or fourth rib. There have been occasional difficulties in finding and aspirating marrow. Such cases have usually proved to be patients with osteosclerosis, sclerosing metastatic tumors, or Paget's disease. Under such circumstances, the operator has often felt that he had pierced the lower sternal plate, but no untoward effects followed.

The case under discussion was one of metastatic tumor producing considerable fibrosis in the marrow. This seems sufficient to account for the difficulty encountered. The fact that the needle reached the heart may have been due to altered mediastinal conditions, the large tumor possibly forcing the heart nearer the sternum than normal.

It might be expected that puncture of the heart, even with an 18-gauge needle, would not result in fatal hemorrhage. However, the needle was fixed in the sternum and the point embedded in the wall of the right ventricle, which apparently resulted in laceration of the myocardium due to the movement of the heart against a fixed, sharp object, the needle point. It is interesting that the laceration did not communicate with the ventricular cavity or with any major branch of the coronary arteries. Apparently, hemorrhage from the myocardium was sufficient to produce fatal tamponade within the course of thirty minutes.

Since this unfortunate accident, whenever difficulty has been experienced, the needle has been withdrawn and a different technique followed. This consists of inserting the needle into the sternum in the same area from below at an angle of 45 degrees. In our experience, whenever difficulty is encountered, the bone has a gritty, porous, corklike feel which makes it easy to penetrate and difficult to determine position. At such an angle, the danger of deep penetration is reduced to a minimum.

### SUMMARY

The second death from sternal puncture recorded in the literature and the first autopsy on such a case is reported. The cause of death was cardiac tamponade, due to hemorrhage from laceration of the right ventricle by the needle.

\*Meyer, Leo M., and Halpein, Jacob: Death Following Sternal Puncture, *Am. J. Clin. Path.* 14: 247, 1944.

## AN INTERMEDIATE TYPE OF OVALOCYTOSIS IN A NEGRO

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**A**N INCREASE in number of oval or elliptical erythrocytes above the Gunther index<sup>4</sup> in healthy individuals is commonly referred to as "ovalocytosis." This phenomenon is considered a nonsex-linked Mendelian dominant character occurring in the white and the Negro races. According to Florman and Wintrobe,<sup>3</sup> these ovalocytes or ellipsoid cells do not undergo a change in shape when subjected to the sealed chamber methods. Hunter and Adams<sup>5</sup> claim that at 54° C. the elliptical cells become discoid in shape. Terry and co-workers<sup>6</sup> and Cheney<sup>1</sup> noted that repeated washing in isotonic saline caused a change of the ovalocytes to round forms. Leitner,<sup>6</sup> according to Wintrobe,<sup>10</sup> found no abnormality in resistance to hypotonic saline, although Stephens and Tatelbaum<sup>8</sup> noted the presence of poikilocytes. Miller and Lucas<sup>7</sup> stress that the elliptical erythrocyte is not a poikilocyte but a congenital anomaly compatible with health. Dameshek<sup>2</sup> described a condition under the name of "Mediterranean Target-Oval Cells Syndrome" in which ovalocytes are part of the peripheral blood pattern. He observed an increased resistance of the erythrocytes to hypotonic saline.

It is the purpose of this paper to report a series of experiments done on a fresh sample of blood obtained by venepuncture from a healthy adult Negro whose erythrocytes were distinctly elliptical (Fig. 1) but which rounded up under sealed conditions (Fig. 2). This change of shape could be reversed. It is surmised that the observations reported here suggest the existence of an, as yet, little investigated "intermediate type of ovalocytosis" belonging in the group of "erythrocyte thinness." There were 86 elliptical cells per 100 erythrocytes, few macrocytes and microcytes, and occasionally a red cell believed to be a poikilocyte. A sealed preparation was made; after fifty-six hours, no sickle cells were present, but practically all elliptical erythrocytes were rounded up. A small sample of blood was repeatedly washed in physiologic saline solution and some rounding up in shape was noted. These washed cells were submitted to the seal test. One preparation was placed in the icebox and another exposed to room temperature. In both preparations the cells had completely rounded up after twenty-four hours. Addition of the donor's plasma to these twenty-four hour preparations caused the cells to assume their former elliptical shape. No increase of poikilocytes was observed. Another sample of washed cells was sealed until rounded up while exposed to room temperature. The seal was then broken and the rounded up erythrocytes returned to their original shape after five hours. Fragility tests on the stock sample and of the washed cells were essentially normal. To a sample of washed and rounded up cells an equal amount of plasma of the same blood type was added; within a few hours the erythrocytes assumed their elliptical shape. A sample of the original blood was permitted to remain in the donor's plasma, and the preparation was stored in the refrigerator for eight days. The majority of the elliptocytes retained their original shape.

The donor's serology was negative. A history of tuberculosis could not be obtained. The blood icteric index was 5 units. Reticulocytes were 1 per cent.

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There was no anemic state. Mean corpuscular diameter of the erythrocytes was 7.7 microns.

#### DISCUSSION AND SUMMARY

The observations presented in this paper seem to suggest that this apparent biologic variable of true ovalocytosis is of an intermediate type and does not necessarily represent a pathologic state. As far as could be determined, this Negro donor enjoyed good health at the time the blood sample was obtained. His erythrocytes were not only distinctly elliptical, but the cells rounded up in the sealed chamber after twenty-four to fifty-six hours. This observation does not support the finding of Florman and Wintrobe, that in the true ovalocytosis the erythrocytes retain their original shape. The change in form would suggest

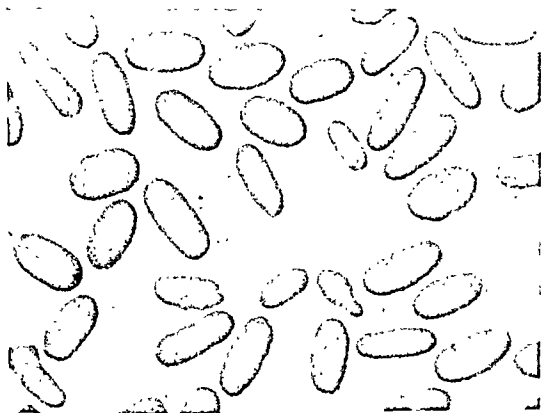


Fig. 1.—Blood film made from specimen obtained by venepuncture. Note the conspicuous elliptical shape of the erythrocytes and the central achromia.

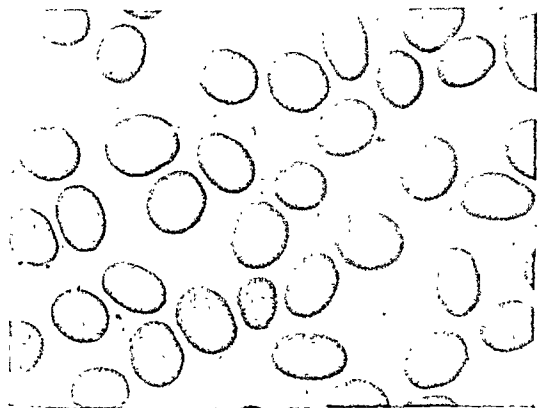


Fig. 2.—Wet sealed preparation. The same blood as shown in Fig. 1 after fifty-six hours. The erythrocytes are either round or distinctly oval. No sickle cells were observed.

that the case presented in this paper is similar to the cases studied by Terry and associates and by Cheney. The fragility test did not reveal any abnormality of the erythrocytes. It is interesting, further, to point out that in film preparations made from the fresh sample of blood obtained by venepuncture, practically all elliptocytes showed a more or less central achromia, but this phenomenon was not observed when the cells were rounded up. No target or sickle cells were observed, but a small number of macrocytes and microcytes and occasionally a poikilocyte were present in the original blood specimen. Their number did not increase in any of the preparations described in this paper. No hemolytic process could be made out at the time the blood sample was obtained. The next of kin of this Negro could not be reached for study purposes.

It is assumed that the case represents an "intermediate type of ovalocytosis." The impression is favored that individuals of this type represent a link between the entities believed by Dameshek to be characterized by erythrocyte thinness, target, elliptical, and oval cells, stippled erythrocytes, hypochromia, and increased resistance to hypotonic sodium chloride solutions.

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#### PRELIMINARY REPORT ON A RAPID METHOD FOR DIAGNOSING SICKLE-CELL DISEASE

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THE following rapid method for the diagnosis of sickle-cell anemia and sickle-cell trait is based on the prompt occurrence of sickle-cell forms when a drop of sickle-positive blood is mixed with a broth culture of a saline extraction of normally excreted human feces. Soon after mixing a droplet of such blood with a drop of the culture on a slide and covering with a cover slip, the accelerated evolution into crescentic forms can be observed in the microscope. The time required for these changes to develop was found regularly to be less than one hour and in some cases only ten to twenty-five minutes. In three recent cases, the characteristic changes appeared after twenty-five, fifty-five, and

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twenty minutes, respectively. The variations in time depend very probably upon both the sensitivity of the blood and the strength of the unknown factor contained within the broth. Thus the long intervals of six to twenty-four or more hours frequently required by the usual sealed wet preparation method, especially in cases harboring the trait only (Wintrobe<sup>1</sup>), are avoided.

Our method has been studied during the past three years on bloods of twenty-three patients with sickle-cell disease and 108 controls. Twenty-two of those with sickle-cell disease were Negroes and one was a white-appearing Nicaraguan boy born of a Negro mother. Of the controls, fifty-eight were Negroes and fifty were white.

*Method.*—A cherry-sized quantity of normal human feces (formed, not diarrheic) is mixed with 5 c.c. of normal saline, stirred with a glass rod to a fine suspension, and filtered. Of the resulting filtrate, 0.1 c.c. is transferred into 5 c.c. normal broth and incubated for twenty-four hours at 37° C. The resultant culture fluid is then ready for use. To examine blood for sickling, a drop of this fluid is placed on a slide and a small droplet of the suspected blood gently mixed into it, covered with a cover slip, and examined microscopically.

We have designated this culture as ferment broth for reasons soon to be understood. Following the twenty-four hour period of incubation, it is necessary to keep the ferment-broth either in the refrigerator or at room temperature but not in the incubator. It is also necessary to renew the culture every few days, either by repeating the above procedure or by transplanting into normal broth.

The changes that can be observed in affected blood are attributed by us to a cell-destructive process, terminating in the appearance of sickled red cells. The process may be termed a hemolysis of sickle-cell type. The damaging factor contained in the broth is very probably of an enzymatic nature. It is suggested that this substance might be identical with, or closely similar to, the so-called "blood group property-destroying factor," or "blood group ferment," as elaborated by Schiff and co-workers.<sup>2</sup> The following similarities are enumerated in support of this assumption:

1. The resemblance between our method of production of the ferment broth and theirs
2. The development of the substance by means of incubation
3. The retention of the substance along with the bacteria upon rigid filtration
4. Its easy transferability from one broth to another
5. The action of the substance upon bloods of all the blood groups
6. Its universal presence in the feces of persons of all the blood groups

It may be further stated that:

1. Sickling of red blood cells never failed to appear with our method in any of the bloods in which sickling had occurred in the ordinary sealed wet preparation. Conversely, we found sickling produced in instances in which the ordinary methods failed. The superiority of the method, therefore, appears to reside not only in its acceleration of the phenomenon, but also in the number of cells involved in the change, as well as in the specificity of action.
2. Sickle-cell anemia blood showed a somewhat more rapid appearance of sickling than "trait" blood with our method. This difference in time, however, does not seem to be of too much significance, since it can easily be attributed to

the high sensitivity of such bloods in crisis and to the smaller number of red cells exposed to the diagnostic agent. We consider it an especially fortunate fact that precisely in the "trait," where the underlying condition cannot even be hinted at in a casual examination of the freshly shed blood, are we enabled to follow microscopically the dramatic development through the several well-definable stages toward the final so-called sickle cell. (A description of these transitional stages will be included in our next communication.)

3. The effective agent in the broth containing the saline extract of feces appears in recognizable strength only after its incubation and is then transferable.

4. The pH of the broth culture, in our experience, does not seem to play a prominent role. After twenty-four hours' incubation, the pH of our broth cultures was found to be around 6.7. Thereafter, the acidity increases whereas the effective strength of the culture decreases.

5. Due to the fact that there is no essential difference in the effects produced by our substance on sickle-cell anemia and sickle-cell trait, we are led to consider both conditions as expressions of the same underlying pathology in varying degree and are therefore using the term "sickle-cell disease" for both conditions.

We will present our detailed findings in a more inclusive report to follow shortly.

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## ALTERATIONS IN THE CONCENTRATION OF THE BLOOD DURING PREGNANCY

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THIS investigation was undertaken in an effort to determine whether or not there was evidence to substantiate the conviction suggested by the work of Dieckmann and his co-workers, that the alterations observed in the blood of pregnant women are due to a dilution of the blood rather than to an actual systemic loss of the blood constituents. That there is a fall in the concentration of the various blood constituents during pregnancy has long been recognized. Killian and Sherwin,<sup>1</sup> for example, in 1921, and also Caldwell and Lyle,<sup>2</sup> found a definitely lowered concentration of nonprotein nitrogen and urea, while Killian and Sherwin also showed a lowered carbon dioxide combining power during the latter months of gestation but failed to find a lowered uric acid. We<sup>3</sup> established ranges for the carbon dioxide content, the oxygen content and capacity, red cell count, cell volume, and hemoglobin concentration of normal blood of pregnant women in contrast to normal blood of nonpregnant women. One of us (J. W. M.),<sup>4</sup> using improved equipment and methods, found a lowered

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range for uric acid. We<sup>5</sup> showed a fall in both serum calcium and phosphorus. Dieckmann and Wegner<sup>6</sup> demonstrate an increase in both blood and plasma volumes, resulting in a fall in cell volume, hemoglobin, and serum protein concentrations per 100 c.c. of blood. This indicates that there is during pregnancy a dilution of the blood which increases the volume and lowers the concentration.

To secure further information on this, we have determined the specific gravity of the whole blood and of the plasma in a series of women throughout pregnancy, following the same individuals so far as was possible. We also determined the red cell volumes. The specific gravity determinations were made by means of the Bimer and Amend falling drop apparatus, as described by Barbour and Hamilton,<sup>7</sup> with the pipette controller recommended by Guthrie.<sup>8</sup> The details of this method have been given by Drew, Scudder, and Rapps.<sup>9</sup> We modified it to the extent of using heparinized whole blood, which could be taken to the laboratory and carefully checked by repeated readings, rather than using finger blood, with no anticoagulant, in the dispensary or at the bedside. Plasma determinations were made on the same heparinized blood specimens after the cells had been spun down for the cell volume determination. The cell volumes are given in per cent of the cells to the volume of the whole blood used after centrifuging it at high speed until there was no further packing of the cells.

The results, given in Table I, show that from the interval of the twenty-seventh to thirty-second week before delivery to that of the fifth to eighth week, there is a small but uninterrupted fall in the specific gravity of whole blood, of plasma, and cell volume. After a rather abrupt fall between the first two intervals recorded, the changes from one interval to another are too small to have statistical significance. The uninterrupted trend, however, which occurs without exception in all three of the factors measured, is most significant. So also is the fall taken as a whole; for example, the averages of the early interval of twenty-seven to thirty-two weeks before delivery compared to those of the five-to-eight-week period give a critical ratio of 5.43 for the whole blood, 5.54 for the plasma, and 4.82 for the cell volumes, showing a very definite statistical significance for all three determinations.

TABLE I  
DILUTION OBSERVED IN PREGNANT BLOOD

INTERVAL (WEEKS BEFORE DELIVERY)	SPECIFIC GRAVITY						CELL VOLUME		
	WHOLE BLOOD			PLASMA			NO.	MEAN	STANDARD ERROR
	NO.	MEAN	STANDARD ERROR	NO.	MEAN	STANDARD ERROR			
27 to 32	29	1.0516	0.000477	31	1.0266	0.000255	31	38.0	0.671
21 to 26	58	1.0504	0.000327	58	1.0257	0.000153	57	35.9	0.498
15 to 20	57	1.0493	0.000316	58	1.0255	0.000221	58	35.2	0.305
9 to 14	63	1.0484	0.000312	63	1.0232	0.000193	63	34.7	0.318
5 to 8	43	1.0482	0.000428	45	1.0248	0.000202	44	34.4	0.327
1 to 4	46	1.0495	0.000395	49	1.0251	0.000114	49	36.1	0.104
7 days post partum	57	1.0527	0.000559	57	1.0271	0.000490	57	39.7	0.751

The lowest values, indicating the period of greatest blood dilution, were obtained for the interval of five to eight weeks before delivery. It should be noted, however, that the true minimum might be somewhat outside this arbitrarily selected interval. The rise to the period of one to four weeks before delivery is again small, but noted in all three factors measured, while that from one to four weeks ante partum to seven days post partum is quite marked. The critical ratios for the entire rise are 6.50 for whole blood, 5.03 for p<sup>1</sup> and 6.47 for cell volume, again all being highly significant.

While one might logically anticipate that this dilution of the blood during pregnancy would be continued until term, the prenatal recovery recorded has also been noted in other determinations. For example, the variation curve of serum calcium and phosphorus reported by us in 1934<sup>2</sup> shows the same tendency. Dieckmann and Wegner<sup>9</sup> also observed that the maximal decrease in hemoglobin concentration occurs between the twenty-sixth and thirty-fifth weeks; that is, five to fourteen weeks before delivery. Dieckmann, however, not only failed to note the prenatal increase during the last four weeks before delivery, but reported the greatest increase in blood volume to be at term. Neither did he observe an immediate post-partum recovery. While we are concerned over this divergence in findings, we feel that we have noted it so distinctly in so many factors that it cannot be ignored. It was apparent not only in the serum calcium and phosphorus, the specific gravity of whole blood, of plasma, and of cell volume, but also in the viscosity of whole blood. Prenatal dispensary admission blood averaged a viscosity of 4.1, with a standard error of 0.049, while seven-day post-partum blood averaged 4.8, with a standard error of 0.215. The critical ratio between these is 3.21, proving that the difference is of definite statistical significance and supporting the observation of immediate post-partum recovery.

We have no explanation for this prenatal recovery, other than that it probably results from a maturity of gestation. There is, however, a most interesting analogy which suggests a possible mechanism for it in the sodium pregnanediol excretion studies made by Bachman, Leekley, and Hirschmann,<sup>10</sup> who find a gradual average increase in the pregnanediol excretion rate from early pregnancy until about two to four weeks before delivery, then a sharp fall until term. Smith, Smith, and Pineus<sup>11</sup> have established a similar prenatal fall in the urinary excretion of estrogens. It would therefore seem possible that hormone regulation of water balance might be responsible both for the blood dilution during gestation and for the slight prenatal concentration during the last few weeks before delivery.

The concentration of the plasma proteins may be obtained from the specific gravity of the plasma according to the formula worked out by Weech,<sup>12</sup> where the plasma protein, in grams per 100 c.c., is equal to the specific gravity of the plasma less 1.0069 times the factor 340.1. We have found this method to be very satisfactory, giving good agreement with other methods of the micro-Kjeldahl type, as, for example, that of Howe.<sup>13</sup> Using this formula we have converted the specific gravity averages of the plasma from Table I to the protein averages for the various intervals recorded, as shown in Table II. This emphasizes the prenatal upward trend and post-partum recovery.

TABLE II  
PLASMA PROTEIN DURING PREGNANCY  
CALCULATED FROM THE SPECIFIC GRAVITY OF THE PLASMA

INTERVAL (WEEKS BEFORE DELIVERY)	NO.	MEAN SPECIFIC GRAVITY OF THE PLASMA	CALCULATED PROTEIN (GRAMS PER 100 C.C.)
27 to 32	31	1.0266	6.70
21 to 26	58	1.0257	6.40
15 to 20	58	1.0255	6.32
9 to 14	64	1.0252	6.22
5 to 8	45	1.0248	6.09
1 to 4	49	1.0251	6.19
7 days post partum	57	1.0271	6.86



## CONCLUSIONS

Evidence has been submitted which indicates that there is a gradual but uninterrupted dilution of the blood from early pregnancy up to a point some five to eight weeks before delivery. From this point on there is a small gradual concentration until delivery, after which the blood quickly returns to non-pregnant levels.

Such a dilution explains the fall in the concentration of the various blood constituents noted by most observers during normal pregnancy.

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## LABORATORY METHODS

### DEMONSTRATION OF THE CORONARY ARTERIAL SYSTEM WITH NEOPRENE LATEX

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**V**ISUALIZATION of the coronary vascular system has been accomplished largely through the use of injection masses or colored fluids forced into the vessels. Mixtures of barium sulfate and gelatin,<sup>1</sup> various colored coagulants, cellulose, and other substances<sup>2-5</sup> have been utilized successfully for such purposes. In some instances, injection of the system with semisolid material facilitated dissection of large or small vessels; in other cases, corrosion specimens of the coronary system (castings of the vessels) were prepared. By such methods, valuable information has been gained concerning the myocardial vascular bed. More recently Batson<sup>6</sup> and Gamble<sup>7</sup> have reported the use of liquid latex as an injection mass in blood vessels. The substance is readily introduced into the arteries and, when solidified, greatly facilitates their identification. Lieb<sup>8</sup> has used neoprene in a study of the vascular tree of the kidney. Her method of perfusing the renal vessels with neoprene appears to provide a simple technique for study of a vascular system either in corrosion specimens or in microscopic section.

This communication describes a method for the preparation of corrosion specimens of the coronary vascular bed in the dog heart.

#### METHOD

*Preparation and Infusion of the Coronary Arteries With Neoprene.\**—For the injection of the coronary vessels with liquid latex, the general principles of Lieb's technique<sup>8</sup> were followed rather closely. Because of the peculiar anatomic conformity of the coronary arteries, it was necessary to devise a special procedure of injection.

1. Dogs anesthetized with sodium pentobarbital were killed by opening the thorax by sternal cleavage, permitting pulmonary collapse.

The heart, lungs, and trachea were immediately excised en masse. The heart was freed by cutting the pulmonary artery and veins and removing the lungs, trachea, esophagus, and other mediastinal tissue. The pericardium was opened and removed. The heart was then rinsed in tap water at room temperature until the chambers were free of gross blood and clots.

2. Glass cannulae were next inserted into the right and left main coronary arteries. The cannulae were introduced through the coronary ostia within the aortic lumen. One ligature of fine string was utilized to tie the cannula in place in each artery and was secured with a bowknot. Later the bowknots could be untied when the cannulae were withdrawn, and then retied in square knots to ligate the vessels. Therefore, it was necessary to use only one ligature about the main coronary stems, of which the left is very short in the dog heart (detail in Fig. 1).

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\*Neoprene latex, Type 571, E. I. du Pont de Nemours and Co.

3. The coronary system was then irrigated with tap water for from four to eight hours. Water was allowed to flow constantly through the heart at a rate of approximately 50 to 75 c.c. per minute.

4. The tubes to the cannulae were clamped, and the heart was placed in the icebox overnight with the cannulae in place and with the tubes and arterial system filled with water. The latter procedure permitted the exclusion of air from the system when neoprene was subsequently infused (see Fig. 1).

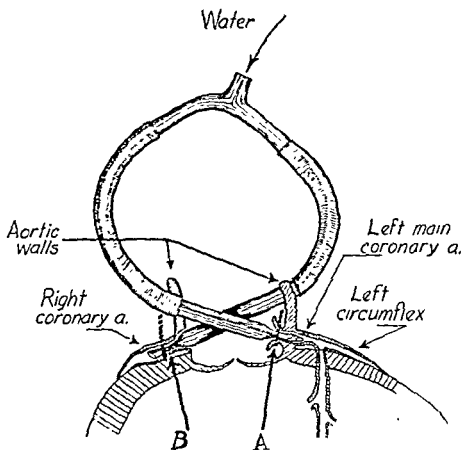


Fig. 1.—Schematic diagram of cannulae in place for irrigation of the heart with water and infusion of the arteries with neoprene latex. Cannulae are inserted into the arteries through the aortic ostia. *A* shows cannula tied into the left main coronary artery; a collar of endothelium about the ostium is dissected up, about which the ligature is secured as shown. This procedure is made necessary by the shortness of the left main coronary. Cannula in the right coronary is secured by a ligature passed around the vessel just without the aortic wall. *B* Before insertion of the cannulae, the tubes are filled with water and allowed to drip slowly so as to exclude air bubbles.

5. After removal from the icebox, the heart was warmed by being placed in warm running water for thirty minutes or more. The tube leading to the cannulae was then attached to a simple pressure flask containing the neoprene latex at room temperature. Neoprene was allowed to flow into each cannula separately, after careful exclusion of air bubbles from the system. Pressures of 150-170 mm. Hg were applied to the system, and the latex was injected for approximately three minutes into each of the coronary arteries, or until all of the superficial vessels seemed obviously well filled. Following injection, the tubes were again clamped; the bowknots securing the cannulae were untied and were quickly drawn up to ligate the vessels when the cannulae were removed, preventing escape of the neoprene.

6. The heart was transferred to a mixture of 4 per cent acetic acid and 1 per cent formaldehyde for from eighteen to twenty-four hours to fix the tissue and to coagulate the rubber.

*Corrosion and Mounting Procedures.*—Because of the "basketlike" character of the entire coronary system when the myocardium and other supporting tissues are removed, and the delicacy of the rubber casting of the vessel system, it was necessary to devise a means of supporting the casting in something like its natural conformity. After many trials the following technique was adopted:

1. When the heart had been fixed in the formalin-acetic acid solution, the auricular and ventricular cavities were filled with neoprene. A small, long-stemmed glass funnel was passed through the superior vena cava and one of the orifices of the pulmonary veins into the chambers and neoprene was poured in slowly. The heart was frequently tipped while filling to remove air bubbles. When the chambers were filled, pledgets of cotton soaked in glacial acetic acid were applied to the orifices to prevent escape of the neoprene.

2. The heart was again immersed in the 4 per cent acetic acid-formalin solution for from eighteen to twenty-four hours to permit hardening of the rubber within the cavities.

3. Care was taken to remove all bits of rubber clinging to the outer surface of the heart which might later adhere to the finished specimen.

4. A small glass rod, pointed at one end, was thrust through both ventricular cavities (piercing the lateral ventricular walls), thus impaling the rubber within the heart. The rod pierced the heart below the auriculo-ventricular sulcus to avoid distorting the principal coronary vessels; it was bent into a wide loop so that the organ could be suspended, apex downward, in a jar containing fluid.

5. The suspended heart was immersed in concentrated commercial hydrochloric acid for two days (at room temperature) to allow digestion of all tissues.

6. The resulting casting of the coronary arterial system is so delicate that care must be taken in removing the acid and digested material. This was accomplished by inserting a small glass tube, connected by rubber tubing to the water faucet, into the acid jar so that its end rested upon the bottom of the jar. Tap water was then allowed to run in slowly for from one to two hours, displacing the acid and digested material. Washing was continued until the casting was free of all particles. The washed specimen was allowed to remain suspended in clear water. Usually glacial acetic acid was added in quantity sufficient to bring its concentration in the bath to about 4 per cent. Such acidification preserves the rubber; the addition of a few crystals of thymol inhibits the growth of mold. If the specimen is to be kept permanently, preservation in Dowie's solution<sup>a</sup> is advised.

#### DISCUSSION

The finished specimen, suspended in fluid, is in conformity to the size and shape of the heart. On close examination, the principal coronary arteries and their tributaries are readily identified, and the ramifications of the smaller vessels may be followed into the delicate tracery of the arterioles and to the capillaries which appear as fine wool. The mass of rubber contained in the ventricular cavities is not visible due to the thick, overlying casting of the ventricular arterial system. The auricular walls are so thin that the vessel network permits the rubber contained in the auricular cavities to be seen.

There was some question as to whether the minute vessels, which, in toto, appear as a mass of spun glass, were in reality castings of the capillaries. The individual strands of this mass are probably hardly visible to the unaided eye. Preliminary studies of the perfusion of neoprene into the capillaries, demonstrated by microscopic sections, has shown that the capillary bed is readily injected with neoprene under conditions identical with those occurring in these experiments. It seems to us probable that most of the capillaries were thus filled.

The infusion of neoprene into each of the main coronary arteries for approximately three minutes (under pressures of from 150 to 170 mm. Hg)

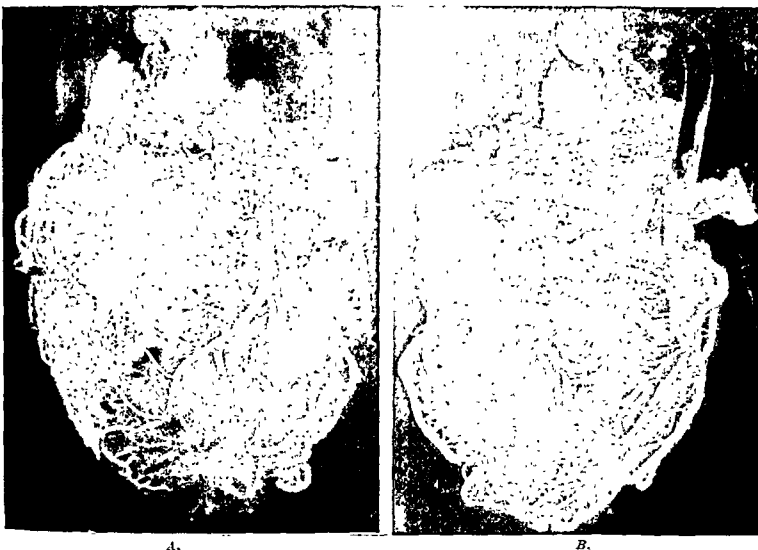


Fig. 2.

Fig. 2.—A shows the finished corrosion specimen of the coronary vessel system of the dog heart in anterior view. The specimen is immersed in fluid. The left descending coronary artery and a portion of the left circumflex coronary artery are readily identified. A small section of the right coronary artery is seen on the extreme right border of the casting (to the left in the illustration). Small branches of the arterial system are visible, finally terminating in the smallest vessels.

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Fig. 3.

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ary was  
ligated at the point indicated by the  
ink line prior to washing the system  
with water and infusion with neoprene.  
The area where vascular filling with  
neoprene failed to occur is shown by  
the depressed area indicated by the  
arrow. Below the point of ligation,  
the left descending coronary slowly  
filled with neoprene by retrograde flow  
from anastomoses with the right coro-  
nary vessel.

appeared to be adequate for reasonably complete filling of the arterial system. When the rubber mixture was permitted to flow into the arteries for longer periods of time, it frequently passed the capillary bed to fill the large cardiac veins. Although demonstration of the cardiac veins filled with latex may be useful in certain types of observation, it was not desired in these experiments. Venous filling was thus prevented by limiting the time of inflow of rubber mixture.

We have found it necessary to keep the specimen suspended in fluid. When removed from the bath, the entire casting collapses into a small, amorphous mass. When replaced in water, the strands of the casting will again separate and float freely so that the larger vessels may be identified, but the most minute strands do not separate from each other and the capillary portions of the exhibit are permanently lost. Therefore, unless a portion of the specimen is to be cut away for special examination, the rubber casting is best kept constantly immersed.

A corrosion specimen of the coronary system may be prepared without attempting to support it by filling the cardiac chambers with latex. The unsupported casting may then be cut longitudinally in any place and floated in water to exhibit the course of blood vessels in plane surface.

Such preparations are useful in demonstrating the extent of the normal coronary vascular system. However, it appears to us that the method may attain greatest usefulness in the study of anastomoses between the principal coronary vessels, and the extent of collateral circulation following coronary occlusion. Our preliminary studies indicate a high degree of anastomosis between the main coronary vessels; the size of infarcts, following the coronary closure, may be dependent upon the presence or absence of such anastomoses (see Fig. 3).

#### SUMMARY

A method is described whereby neoprene latex is infused into the coronary vessels of the dog heart. Corrosion specimens, employing concentrated hydrochloric acid, may be made which appear to preserve all details of the coronary arterial system lumina including the capillaries. The cardiac chambers may be filled with liquid latex and the whole organ suspended by a glass hook to support the vascular casting when the myocardium is digested away. The finished specimen is kept immersed in fluid so that the minute strands are separated and details of the coronary vascular system may be seen.

The authors are indebted to Dr. Robert A. Moore, Department of Pathology, for many helpful comments and for the suggestion that neoprene be used in these experiments. The Rubber Laboratories of E. I. du Pont de Nemours and Co. kindly furnished the neoprene latex used in these experiments.

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# A RAPID PRESUMPTIVE COMPLEMENT FIXATION TEST FOR SYPHILIS

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THE rapid presumptive complement fixation test to be described is useful in weeding out possible cases of syphilis in physical fitness groups, in pre-transfusion donor testing, and in similar situations where any rapid presumptive test is needed. It does not in any sense replace the standard Kolmer, Kline, Hinton, Eagle, or Kahn tests and should be checked by one or more of the standard tests as soon as practicable. The reaction depends on the native complement and the native hemolysin against guinea pig erythrocytes present in the examined blood. Either or both of these may occasionally be weak or absent, in which case the test yields a false positive result. However, since a diagnosis is not made on this method of testing, an occasional false positive reaction is inconsequential. The test is simple and requires very little equipment not usually found at medical stations. (An orange crate will house four pigs, a month's supply, if an animal is sacrificed at each bleeding.)

## Method.—

1. Transfer 1 c.c. of freshly drawn venous blood into a small tube containing 0.5 c.c. of Kolmer antigen ( $\frac{1}{75}$ \*) and shake vigorously.
2. Place the tubes in a water bath at 37 to 39° C. for one-half to one hour.
3. Remove tubes from the water bath and cut the clot loose if it is adherent to the tube wall.
4. Centrifuge the tubes until the supernatant stratum is free from erythrocytes.
5. Fill a white blood cell-counting pipette to the bulb with the supernatant, erythrocyte free, serum-antigen mixture, continue filling the pipette to the "11" mark with 1 per cent washed guinea pig erythrocytes, and mix.
6. Incubate under desk lamp at 37 to 38° C. for fifteen minutes.

A hemolytic control tube is not routinely used since all the tests showing incomplete hemolysis are checked routinely by standard methods, regardless of the cause of inhibition. If desired, a control may be set up by substituting 0.5 c.c. of 0.85 per cent saline solution in step 1 for the 0.5 c.c. of antigen mixture. This will show if the natural complement-hemolysin content is adequate. Equipment required for performance of the test is illustrated in Figs. 1 and 2.

A negative test shows complete hemolysis of the guinea pig cells in the pipette bulb; the white bead appears sharp in outline. A strongly positive test shows no hemolysis, the bulb contents being opaque and the agitating bead indistinct. After the cells sediment to the bottom of the bulb, the supernatant fluid is water clear. Weakly positive tests show partial but not complete hemolysis. After standing some time, the sedimented cells have a tendency to agglutinate. Rabbit erythrocytes are not suitable because of the lower natural hemolysin content of human serum against them and a much greater tendency

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\*We have tried several Kolmer antigens, some standard stock antigens furnished by the U. S. Navy, and some of our own extraction. The stock antigen is of such strength that if employed in the standard Kolmer test it is used in .5 c.c. quantities of a 1/500 dilution. It is mixed by placing .2 c.c. of stock antigen into a clean, dry, Pyrex, four-ounce, small-neck nursing bottle and adding drop by drop 15 c.c. of isotonic saline with vigorous shaking.

to agglutinate. Washed 1 per cent guinea pig cells (three washings) suspended in 0.85 per cent saline kept at 8° C. are usable for a week. We have checked this test against a standard Kolmer, a standard Kahn, and clinical history and diagnosis. A few false positive reactions are encountered, but we have not missed a full  $\pm$  plus reaction, and this test compares favorably with other tests in weakly positive cases having a lower reagin content. There is at least not more conflict than we find between the Kolmer and Kahn tests as done routinely. Since there is so little processing of the few reagents, technical errors are minimal.

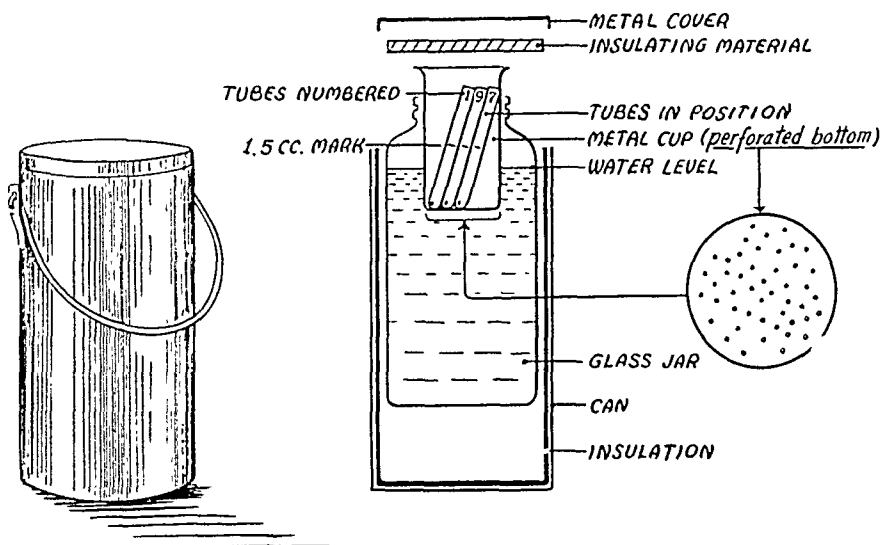


Fig. 1.—A portable, efficient water bath can be made with a fruit jar and a perforated can to fit in the top and with some sort of cloth or paper insulation.

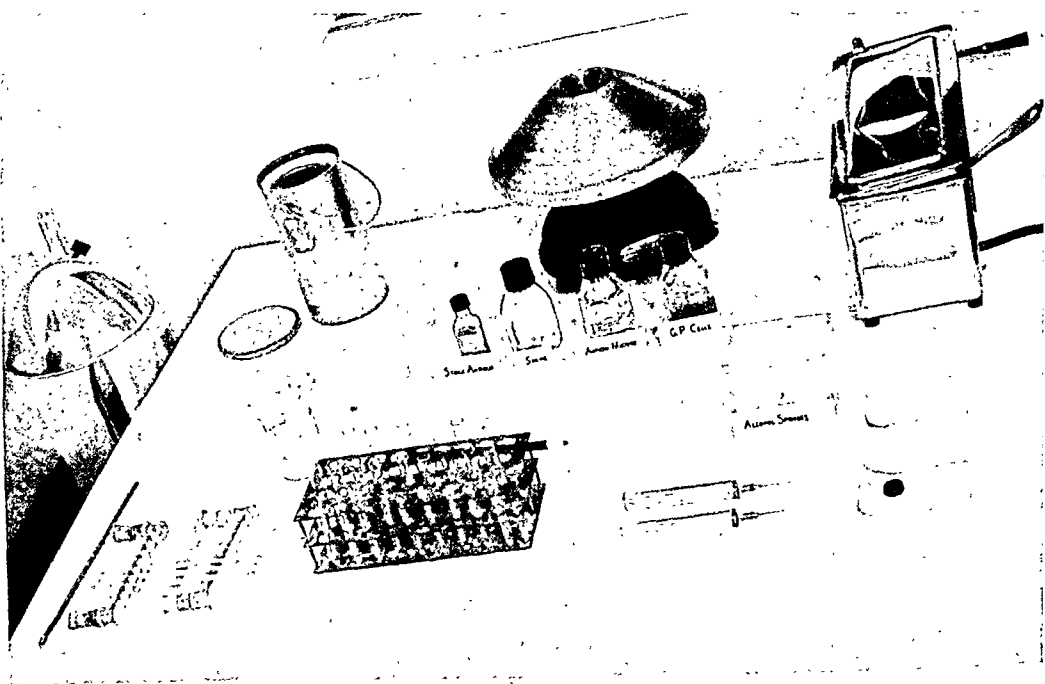


Fig. 2.—The setup conveniently placed on a 32 by 46-inch typewriter desk top with room to spare. The larger tubes in the test tube rack are for the purpose of collecting additional blood for follow-up serology or other tests.



We have completed 724 tests of blood obtained from patients in the skin, surgical, and medical wards with various diagnoses, including many tropical diseases. The patients were almost all men between 18 and 45 years of age—white, Negro, and Oriental. Each presumptive test was controlled by a standard Kahn and a quantitative Kolmer test. In this group were 124 cases in which there was a strongly positive reaction in all three tests. There have been four strongly positive presumptive tests which were negative by the standard tests. One of these was in a patient with a skin lesion diagnosed papulo syphilodum on admission and one was in a patient with a diagnosis of hookworm, who gave a history strongly suggestive of yaws. In the other two cases the patients showed no clinical evidence of syphilis and gave negative histories. Emulsions from lymph glands of these patients injected into testes of rabbits produced no lesions (still under observation). Two early cases were positive by the presumptive test eight and ten days, respectively, before the standard tests were positive. Six cases were not as strongly positive as the standard tests. Eighteen patients showed doubtful reactions, which were negative by the standard tests.

Acknowledgment is made of technical assistance of Nathan T. Hodges, Ph.M. 1/c, U.S.N.R., who independently conducted the control Kolmer and Kahn tests and in other ways assisted in the setup.

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## THE USE OF PLASTICS TO REPLACE COVER GLASSES IN MICROSCOPE SLIDE PREPARATIONS

EUGENE DE ANGELIS, M.D.  
MORGANTOWN, W. VA.

THIS paper deals with a method of applying plastics to microscope slide preparations, the main purpose being to eliminate the routine use of cover glasses. Persons familiar with these preparations realize the tedium of cleaning and placing cover glasses. This fastidious, slow, and therefore, relatively expensive procedure has often been a source of annoyance to me, and has led to the present study as a means of eliminating it. The object is to develop a multiple slide procedure which can be carried on in a shorter time and at a decreased cost.

### MATERIALS

The materials selected were plastics which were especially recommended for coating purposes.

The desired properties were: (1) strong adhesion to glass and tissue, (2) maximum optical transparency, (3) resistance to abrasion and to common solvents, (4) slow or negligible deterioration by light and chemicals, and (5) ease of application.

Several types of plastics were tried in order to find suitable ones. The nitrocellulose plastics offered some desirable properties, but these were offset by an observed poor resistance to ultraviolet radiation. Several alkyd resins were found unsatisfactory. The two plastics which appeared most satisfactory in this preliminary work were Acryloid B-72\* and Vinylseal.†

From the Department of Pathology, West Virginia University Medical School.  
Received for publication, Feb. 25, 1945.

\*Obtained from The Resinous Products & Chemical Co., Philadelphia, Pa.

†Obtained from The Bakelite Corporation, New York, N. Y.

Acryloid B-72 is an acrylic resin obtained from the manufacturer in 40 per cent solution of toluol. Its chief disadvantage is a pronounced solubility in xylol and other common solvents, although its coating and optical properties were found to be excellent. This plastic was superior to the others used in this experiment, and its resistance to ultraviolet light is enormous. Solutions made thinner with toluol can be used for blood, bacterial, and exudate smears.

Vinylseal is a vinyl acetate resin in methyl acetate (or other solvents). This is rather resistant to xylol, but it does not have the coating qualities of the Acryloid B-72, and its use requires much greater care. Further work with vinyl resins should reveal definite possibilities. At the present time I do not use vinyl resins.

It should be stated that an exhaustive study of a greater number of plastic substances should bring to light materials which have even more desirable properties than have those upon which this report is based. Rather than to make a detailed study of all of the available plastics, it was considered more desirable to submit this preliminary report in order to stimulate further investigation.

#### METHOD

The technique is applicable to one or any number of slides. A proper multiple slide holder, such as the Fisher-Brubach holder, is used if several slides are to be prepared.\* After the preparation has been stained and passed through the alcohols and xylol, it is dipped slowly into the plastic solution and allowed to remain for about thirty seconds. It is then slowly lifted out, supported in a rack over the solution at an angle so that the excess plastic drains off. This procedure is usually repeated once, and when the plastic ceases to flow, the holder is hung on a rack to permit drying of the slides. The number of applications of this plastic depends upon the thickness desired, and this in turn depends upon the thickness of the section.

After complete drying, the slides may be examined under the microscope with little risk of damage. However, if the slides are placed in an ordinary drying oven and baked at 140° C. for one hour, the plastic, on cooling, will be very hard and much more resistant to abrasion. This procedure must be followed if the slides are to be filed.

#### APPLICATION

The chief purpose of this technique is its application to fixed tissue preparations in order to eliminate cover glasses for routine use. It may also be applied by using thinner solutions of plastic to various preparations such as blood, bacterial, and exudate films for the purpose of providing protection and making a better-appearing slide, although obviously this is unnecessary. I have, however, used it in making preparations of parasite ova, cysts, and the like, and find it extremely simple and superior to many other methods. In this case the concentrated fecal material containing ova or cysts is smeared on the slide, allowed to dry, and dipped into the plastic solution. An alternate method consists of allowing the plastic to flow over the film and to dry. This method also probably can be used in replacing the cumbersome Canadian balsam technique in making Golgi preparations for nerve tissues.

There are certain possible disadvantages, the elimination of which will require additional work. In regard to Acryloid B-72 preparations, one may be

\*A special tray, slide holder, and rack are being devised for this technique.

of importance. This plastic is fairly soluble in xylol and certain other solvents, and care should be taken to see that such solvents do not come into contact with the slide. However, if this should occur, a new surface can readily be applied. Immersion oil has no adverse effect; in the event that it should dry on the slide, it may readily be removed by adding more liquid oil and wiping.

The advantages are obvious. This procedure would completely eliminate cover glasses in routine preparations, decrease cost, increase speed of preparation, and simplify multiple preparations, such as serial sections of embryos, brain preparations, and the like. Labels, if applied to plastic with a solvent, become extremely adherent. If valued slides were to fade in time, they could be restained and resurfaced with ease. In addition, application is almost universal to all types of slide preparations.

Slides kept so far for more than six months look exactly as they did the day they were prepared, even though they have been given rough treatment.

#### SUMMARY

This paper presents a new technique; namely, the application of plastics to replace routine use of cover glasses in microscope slide preparations.

The author wishes to express his sincere and deep appreciation for the invaluable aid rendered by Dr. Howard Perry Simons, Associate Professor of Chemical Engineering, West Virginia University. He not only helped with his knowledge of plastics, but also corrected the original manuscript.

The Resinous products & Chemical Company and the Bakelite Corporation kindly and generously donated some of the plastics which made this work possible. To these companies the author is greatly indebted.

Acryloid B-72 is an acrylic resin obtained from the manufacturer in 40 per cent solution of toluol. Its chief disadvantage is a pronounced solubility in xylol and other common solvents, although its coating and optical properties were found to be excellent. This plastic was superior to the others used in this experiment, and its resistance to ultraviolet light is enormous. Solutions made thinner with toluol can be used for blood, bacterial, and exudate smears.

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There are certain possible disadvantages, the elimination of which will require additional work. In regard to Acryloid B-72 preparations, one may be

\*A special tray, slide holder, and rack are being devised for this technique.

devoted to the natural history of antigens, in which approximately equal attention is paid to the chemistry, and to the genetic and phylogenetic relationships, of animal and bacterial antigens.

Other topics, such as physico-chemical and theoretical considerations of the immune reactions and the origin of antibodies, are treated in a frankly synoptic and uncritical manner, as is appropriate to the nature of the work, which is first of all that of a great experimenter. These topics are nevertheless faithfully noted in the text and are included in the generally complete bibliography.

The attention of the physician may be called especially to the chapter entitled "Hypersensitivity to Substances of Simple Composition" (pages 197-207), which includes a review of the experimental demonstration, principally by Landsteiner and Jacobs, and Landsteiner and Chase, of the immunologic mechanism responsible for at least one kind of contact dermatitis.

The book closes with a brief but characteristically lucid chapter by Linus Pauling on the structure of molecules and the nature of the known intermolecular forces. This is concluded by a very clear picture of the way in which large molecules may be understood to combine both firmly and specifically as a result of the coordination of many weak interatomic bonds operating in the area of contact between complementary surfaces.

A. D. Hershey.

**The Practice of Medicine.** By *Jonathan Campbell Meakins, M.D., LL.D.*, Brigadier, Deputy Director General of Medical Services, Royal Canadian Army Medical Corps; Professor of Medicine and Director of the Department of Medicine, McGill University; Physician-in-Chief, Royal Victoria Hospital, Montreal; Formerly Professor of Therapeutics and Clinical Medicine, University of Edinburgh; Fellow of the Royal Society of Physicians, London; Fellow of the Royal College of Physicians, Edinburgh; Honorary Fellow of the Royal College of Surgeons, Edinburgh; Fellow of the Royal College of Physicians, Canada; Fellow of the American College of Physicians; Honorary Fellow of the Royal Society of Medicine. Fourth edition. The C. V. Mosby Company, St. Louis, Mo., Price \$10.00. Cloth with 1,444 pages and 517 illustrations including 48 in color.

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## ANNOUNCEMENT

In compliance with the directives imposed by the War Production Board limiting the amount of paper consumed in the production of this JOURNAL, the publishers find it necessary to change the format. As soon as these restrictions are lifted the original format will be restored. Even though the number of pages has been reduced, the actual content of the JOURNAL has not been decreased to any appreciable extent.

# NUTRITIONAL SURVEY IN NORRIS POINT, NEWFOUNDLAND

J. METCOFF, M.D.,\* BOSTON, MASS., GRACE A. GOLDSMITH, M.D.,† NEW ORLEANS, LA., A. J. McQUEENEY, M.D.,\* BOSTON, MASS., R. F. DOVE, M.D.,\*\* NORRIS POINT, Nfld., ELLEN McDEVITT, A.B.‡ NEW YORK, N. Y., MARGARET A. DOVE, B.Sc.,‡ NORRIS POINT, Nfld., AND F. J. STARR, M.D.,\* BOSTON, MASS.

## INTRODUCTION

NUTRITIONAL studies have suggested that malnutrition is an extensive and important problem in Newfoundland. Little<sup>1, 2</sup> described beriberi in Newfoundland, stressing in particular its occurrence in association with a diet restricted largely to white flour. Appleton,<sup>3</sup> Aykroyd,<sup>4, 5</sup> Mitchell,<sup>6</sup> Vaughn and Mitchell,<sup>7</sup> and Steven and Wald<sup>8</sup> studied dietaries and deficiency diseases in Newfoundland and Labrador. A high incidence of beriberi, vitamin A deficiency, dental caries, spongy hypertrophied gums, and chronic malnutrition was noted. Olds<sup>9</sup> and McDevitt and co-workers,<sup>10</sup> working in widely separated areas of Newfoundland, found low levels of ascorbic acid in the blood in a high percentage of persons tested. The most extensive survey of nutrition in Newfoundland is the recent report by Adamson and associates.<sup>11</sup> These workers examined 868 people and did certain chemical analyses on the body fluids of nearly half of the subjects seen. Deficiencies of vitamin A, riboflavin, and ascorbic acid were frequent.

The present report describes a nutritional survey at Norris Point, during June, 1944, made with a twofold purpose: (1) rapid assessment of nutritional status by examination of a weighted population sample; (2) establishment of a baseline from which the future nutrition of the same or similar samples can be judged. It was particularly desirable to establish this baseline, for in the near future enriched flour was to be introduced into Newfoundland.||

Norris Point is a west coast fishing and logging village of approximately 100 families. It is typical of many such villages, the inhabitants of which make up a large part of the total population of Newfoundland. Many factors favor the existence of malnutrition: (1) geographic isolation; (2) primitive transportation; (3) minimal agricultural production on leached marginal land by obsolete wasteful methods; (4) inadequate educational facilities; and (5) poor housing, food storage, and cooking facilities.

The Scotch-English stock at Norris Point, which comprised the sample, had all lived under such influences for at least two years, many for generations. The incidence of blood relationship was high. Certain colloquialisms of speech, together with reticence and shyness, particularly in the presence of strangers, made accurate history-taking difficult.

This survey was made possible by a grant-in-aid from the Williams-Waterman Fund, Research Corporation, New York, N. Y.

Received for publication April 2, 1945.

\*Department of Nutrition, Harvard School of Public Health, Department of Biological Chemistry, Harvard Medical School, and the Medical Clinic of the Peter Bent Brigham Hospital.

†Department of Medicine, School of Medicine, Tulane University of Louisiana.

‡Bonne Bay Cottage Hospital, Department of Health and Welfare; Senior Medical officer, Bowater's Newfoundland Pulp & Paper Mills, Ltd., Corner Brook, Nfld.

§Bonne Bay Cottage Hospital, Department of Health and Welfare.

||Department of Medicine, New York Post-Graduate Medical School and Hospital, Columbia University.

By order of the Newfoundland Government and on recommendation of the Council on Nutrition of the Newfoundland Medical Association, all flour entering the country after July 1, 1944, is to be enriched to new American standards.

## MATERIAL AND METHODS

The population sample was selected from groups known to be particularly susceptible in regard to clinical deficiency disease, notably pregnant and lactating women and rapidly growing children. Such groups may be considered as a sample so weighted as to uncover nutritional deficiency if any exists. This sample is not representative of the general nutrition of the community but rather is likely to approximate the poorest nutrition. It was felt that if these groups showed no evidence of malnutrition, no great amount of malnutrition would be present in the population at large.

The following criteria were established. The family was made the unit of study, and only those families in which there had been a pregnancy within the previous two years were included. In addition to the women of such families, their children between the ages of 3 and 6 and 10 and 14 years were studied, while other children were excluded. Husbands in such families were examined when available but often were absent in fishing or lumbering industries. The total group was homogeneous with regard to socioeconomic status. Of the 40 families in Norris Point which fulfilled these criteria, all but one agreed to take part in the survey. The sample of thirty-nine families had the following composition: women, 39; children 3-6 years of age, 34 (21 boys, 13 girls); children 10-14 years of age, 24 (10 boys, 14 girls); men, 6. Of the women examined, 6 were pregnant and 14 lactating. Ten additional men seen in the clinic were included in the survey.

The 113 individuals were examined by a survey team of 6 members in a period of ten days. The local physician administered the project and acted as liaison officer in the selection of the sample. Three physicians obtained medical histories and conducted the physical examinations. All positive physical findings were observed by all three physicians and recorded only if there was a majority agreement. Two research associates obtained nutritional histories and were responsible for photography and assistance in laboratory work.

The complete examination consisted of five parts: (1) A nutritional history was taken, consisting of a record of all nourishment ingested in the previous twenty-four hours. A complete list of all available foods was used to remind the individuals of foods they may have eaten and to uncover any peculiarities in the diet on the day of the test. At the same time the frequency of intake of protective foods per day and week was recorded. (2) A detailed medical history was taken, and this was later supplemented by the local hospital records. (3) A thorough and extensive physical examination was made with special attention given to eyes, mouth, skin, and neurological findings. Height and weight were recorded on all individuals, and in the case of children the intercrural diameter was measured. (4) Photographs consisted of two types: (a) color films of all lesions; (b) nude full-length black and white photographs taken of most children at a fixed distance, against a standard measuring scale. (5) Laboratory tests undertaken included the usual routine urinalysis for qualitative detection of sugar, albumin, and cellular material, and the determination of hemoglobin and total plasma protein from a 2 to 3 ml. sample of venous blood. The gravimetric copper sulfate method of Phillips and associates<sup>12</sup> was used. The recording was so planned that all necessary data could conveniently be checked off or indicated on 5 by 8 filing cards. Diagrams of body surface, face, and tongue were included on these cards and proved to be particularly useful in indicating the exact location and extent of lesions and in recalling the various cases later when the data were assembled.



## FINDINGS

*Nutritional History.*—The diets of all of the families examined showed remarkable similarity. Children, as soon as they are weaned, receive the same food as adults. A typical daily menu is given in Table I. The main protein foods are fish (largely salt cod and herring in the winter), salt pork, beef (with much fat), dried beans, and peas. Occasionally some fresh meat is consumed when wild game can be obtained or when a sheep, cow, or pig is slaughtered. Lack of storage facilities makes the supply of fresh meat largely dependent on the weather. The supply of fresh fish (cod, salmon, lobster, herring, tuna, trout, caplin) is dependent on the run in a particular year. Margarine, fortified with 6,000 I.U. of vitamin A per pound, is used as practically no butter is available. Fresh vegetables are not available from October to July, and during this period root vegetables, including potatoes, turnips, carrots, parsnips, and beets, are used in addition to cabbage, which may be either fresh or salted. Potatoes are eaten about twice daily unless the family supply is exhausted before the winter ends. A few families eat green leafy vegetables in the summer months.

TABLE I. TYPICAL DAILY MENU

<i>Breakfast</i>	<i>Dinner</i>	<i>Supper</i>
Porridge with milk (30 to 40 ml.) and sugar or molasses	Fish (salt or fresh), fried or boiled	Same as noon meal—leftovers reheated
or	Potatoes	Bread, biscuits, or cake
Baked beans	or	Tea
Bread with margarine, jam, or molasses	Hash (salt meat and potato)	<i>Occasional Foods</i>
Tea	or	Eggs, two or three times weekly
<i>Between Meals</i> <i>(Two or Three Times Daily)</i>	Thick soup (stew)	Dried peas, cooked tomatoes with macaroni, once weekly
or	or	Evaporated milk (1 to 2 tsp.) in tea
Bread, biscuits, jam, cake, or cookies	Boiled dinner (potatoes and turnips; occasionally cabbage and carrots; salt or fresh meat if available)	
Tea	Bread with margarine, jam, or molasses	
	Tea	

The preparation of vegetables makes it doubtful whether much of the vitamin content is retained. Both meat and vegetables are cooked for long periods of time. The supply of fruits is limited to a few oranges during the spring and summer months, apples and dried fruits, which are used sparingly except in jam or deserts, and wild berries, also used in jam. In most instances the bread is homemade from unenriched white flour. Breakfast cereals are usually cream of wheat or rolled-oat porridge cooked overnight and served with sugar or molasses. The consumption of milk, even among children, is low. Of the 54 children studied, 4 drank no milk, 21 used milk only with tea or cereal, 21 used from one-half to one cup a day, and only 8 drank over one cup of milk daily.\* A small number of persons had food prejudices, tomatoes, dark bread, and milk being the most frequent.

An attempt was made to calculate the daily intake of various nutrients from the dietary histories, using the tables of the Committee on Food Consumption

\*During the winter months all schools that apply are supplied with powdered milk free by the government for the children in the school.

TABLE II. NUTRIENT INTAKE ESTIMATED FROM DIETARY HISTORY

	FAM- ILY	CASE	CAL- ORIES	PRO- TEIN (GM.)	CAL- CIUM (GM.)	IRON (MG.)	VITA- MIN A (I.U.)	ASCOR- BIC ACID (MG.)	THIA- MINE (MG.)	RIBO- FLAVIN (MG.)	NI- ACIN (MG.)
Adult women	27	62	2000	69	0.4	11	1,300	41	0.8	0.9	11
	30	73	2200	64	0.6	10	1,600	35	0.9	1.1	8
Children 10-14 years of age	28	66	1600	51	0.3	6	600	18	0.5	0.7	7
	23	50	1800	52	0.4	11	2,000	41	1.1	1.0	10
Children 3-6 years of age	30*	74	1400	42	0.6	6	1,200	26	0.6	1.1	5
	12†	25	1300	42	0.3	4	700	6	0.4	0.7	4

\*No cod-liver oil.

†Cod-liver oil (?).

TABLE III. SYMPTOMS SUGGESTIVE OF NUTRITIONAL DEFICIENCY

SYMPTOMS	NUMBER OF CASES *		
	WOMEN (39)	CHILDREN (24) 10-14 YR.	MEN (16)
Eyes			
Burning or itching	20	3	5
Photophobia	17	4	3
Lacrimation	14	4	7
Visual fatigue with blurring	7	1	1
Redness	6	0	4
Discharge	3	0	2
Night blindness	5	0	3
Snow blindness	2	2	9
Lips, tongue, gums			
Burning painful tongue	14	1	2
Cracked painful lips	8	7	6
Sores at angles of mouth	3	0	0
Bleeding gums	20	6	3
Cardiovascular and respiratory			
Exertional dyspnea	14	0	3
Palpitation	12	0	3
Precordial pain	3	0	0
Edema	1	0	0
Frequent upper respiratory infec- tions	5	0	3
Gastrointestinal			
Hemorrhoids with bleeding	10	1	2
Constipation	8	3	1
Anorexia	6	0	3
Indigestion	13	1	7
Chronic diarrhea	1	1	1
Weight loss	2	0	1
Neuromuscular skeletal			
Numbness and tingling	11	1	7
Syncope or dizziness	10	1	3
Muscle weakness	6	0	2
Burning feet	2	0	3
Leg cramps	13	1	4
Painful joints	5	0	1
Skin			
Easy bruising	15	0	3
Burning	2	0	0
General			
Nervousness and irritability	14	1	3
Easy fatigability	10	0	2
General weakness	3	0	1
Anxiety	4	0	0
Insomnia	1	0	0
Depression	1	0	0

of the National Research Council. This was possible in only a small number and even in these the figures represent rough approximations. Findings representative of the dietary data are given in Table II. The figures given for the ascorbic acid content of the diet are probably much too high because of losses in preparation of food. Since the dietary calculations were subject to such gross error, they were not used in the appraisal of nutritional status.

*Symptoms.*—The pertinent symptoms are listed in Table III for all groups except children between the ages of 3 and 6 years, in which group symptoms could not be evaluated. Burning and painful eyes, visual fatigue, photophobia, and lacerimation were frequent complaints. All persons were familiar with the terms night blindness and snow blindness, and of the 79 adults and older children questioned, 8 gave a history of night blindness and 13 of snow blindness. Soreness of the tongue had been noted by 17 persons and cracked painful lips by 21. Bleeding gums had been present in 29 individuals, more than one-third of the group. There was a high incidence of exertional dyspnea and palpitation as well as complaints of numbness and tingling of the extremities, syncope, and dizziness. Cramps in the legs, nervousness and irritability, and easy fatigability were common complaints of the women. "Indigestion" or dyspepsia and constipation were frequent gastrointestinal symptoms, and it is of interest that Steven and Wald<sup>6</sup> commented on a condition known as "Newfoundland stomach," which was characterized by these symptoms in association with many vague general complaints. They suggested that the condition might be related to thiamine deficiency.

As would be expected, the various symptoms were more prevalent in the adults.

*Physical Findings.*—The findings on physical examination are given in Tables IVA and IVB. Various eye signs were quite prevalent, particularly conjunctival thickening and injection and Bitot's spots. The designation "marked thickening" includes instances of pterygium and pinguecula. The term "conjunctival injection" includes an increase in the blood vessels along the equator as well as gross injection of the eyeball. An invasion of the cornea by blood vessels from the circumlimbal plexus, visible with a hand lens, was designated as a "pannus." Bitot's spots were diagnosed in 50 per cent of the adults on the basis of well-defined white or yellowish pearly elevated spots located on the scleral conjunctiva, usually near the corneal limbus. The incidence of all of the eye signs increased with the age of the individuals examined, and injection of the conjunctiva was much higher in adult men than in adult women.

One of the most frequent signs observed was some change in the papillae of the tongue; this was present in over 75 per cent of the 113 persons examined. Slight changes included either minimal enlargement and redness of the fungiform papillae or slight atrophy of the papillae at the tip or sides of the tongue. Moderate to marked changes occurred in more than 20 per cent of the men and children and in more than 50 per cent of the women. A magenta-colored tongue was observed in 12 per cent of the men, 21 per cent of the children, and 51 per cent of the women. Angular stomatitis was found in 12 per cent of the men, 19 per cent of the children, and 28 per cent of the women.

Lesions of the teeth and gums were very common. Dental caries was found in over 80 per cent of persons, being high even in children 3 to 6 years of age; however, caries and missing teeth cannot be evaluated by American standards as there is no preventive dentistry in Norris Point, and when symptoms are severe or caries is extensive, extraction of teeth is the rule. The incidence of periodontal disease was high and increased with age. Gums which bled on +

TABLE IVA. PHYSICAL SIGNS SUGGESTIVE OF NUTRITIONAL DEFICIENCY

SIGNS	NUMBER OF CASES			
	WOMEN (39)	CHILDREN (58)		MEN (16)
		3-6 YR. (34)	10-14 YR. (24)	
Eyes				
Conjunctiva thick—pigmented				
Slight	2	5	5	1
Moderate	17	4	3	9
Marked	19	1	2	6
Bitot's spots	19	1	4	8
Conjunctival injection	21	6	3	14
Circumcorneal injection	9	5	5	9
Pannus	4	0	1	3
Xerosis	0	0	1	0
Tongue				
Papillae, atrophy and/or hypertrophy				
Slight	10	15	12	7
Moderate	8	8	3	3
Marked	15	3	2	1
Magenta color	20	7	5	2
Red tip and/or sides	5	7	3	5
Fissures	17	0	2	3
Erosions or ulcers	3	1	0	0
Serrations and swelling	1	0	0	2
Lips and mouth				
Angular stomatitis	11	6	5	2
Cheilosis	2	3	1	1
Pallor of mucous membrane	6	4	0	0
Ulcers of mouth	2	1	0	0
Teeth and gums				
Caries				
Slight	7	9	15	4
Moderate	10	6	1	4
Marked	17	11	6	5
Edentulous	5	0	0	2
Periodontal disease				
Slight	3	5	8	3
Moderate	15	0	0	8
Marked	10	1	1	3
Bleeding gums	8	1	3	4

ing were present in 21 per cent of the women, 25 per cent of the men, and 7 per cent of the children.

On examination of the skin, there were numerous abnormal findings. Changes in the sebaceous glands of the face of the type often seen in ariboflavinosis were common. Follicular hyperkeratosis was found in 36 per cent of the women, 48 per cent of the children, and 50 per cent of the men. This term includes mild "permanent goose flesh" involving merely the arms or thighs, as well as more severe and extensive lesions. Petechiae, purpuric spots, and general pallor were rarely observed.

Objective neurological findings included calf muscle tenderness and reflex and sensory disturbances in the feet and legs, although these findings were not so frequent. It is interesting to note the frequency with which systolic heart murmurs were encountered. Systolic heart murmurs have often been described in beriberi. The skeletal abnormalities which were suggestive of rickets are given at the end of Table IVB. The height of each person was recorded and the weight was taken without shoes but with the person wearing either underclothing or a bathrobe. The adults were, in general, of short stature and thin. Only one woman of the group was definitely obese. The children appeared to be under-size both in height and weight, and comparison with standards of healthy white American children<sup>13, 14, 15</sup> indicated a trend in this direction. In Fig. 1 are

TABLE IVB. PHYSICAL SIGNS SUGGESTIVE OF NUTRITIONAL DEFICIENCY

SIGNS	NUMBER OF CASES			
	WOMEN (39)	CHILDREN (58)		MEN (16)
		3-6 YR. (34)	10-14 YR. (24)	
Skin—facial				
Seborrhea, nasolabial	7	7	5	1
Seborrhea, face, ears	5	4	2	0
Erythema	5	2	1	0
Skin—general				
Follicular keratosis	14	16	12	8
Dryness and scaling	6	1	4	0
Acneiform eruption	4	0	1	0
Thick and pigmented over elbows and knees	12	2	7	2
Purpura and petechiae	4	0	0	0
Bluish-red, cold extremities	5	3	4	8
Neuromuscular				
Calf tenderness	5	0	0	3
Absent knee jerk or ankle jerk	3	1	0	1
Plantar dysesthesia	3	0	0	2
Motor weakness (legs)	1	0	0	0
Absent or decreased vibratory sensation	2	0	0	3
Cardiovascular				
Systolic murmur	23	8	1	3
Pitting edema	0	0	0	0
Skeletal				
Frontal or parietal bosses		10	0	
Protuberant abdomen		7	0	
Harrison's groove		6	2	
Knock-knees		6	0	
Wrist joints enlarged		2	0	
Enlarged costochondral junctions		2	6	
Winged scapula		1	3	
Flaring ribs		1	1	
Scoliosis		0	1	

given the findings for boys. It can be seen that the weight of the Newfoundland boys aged 3 to 6 years was within the normal range of the American group but that 8 of 19 children were of shorter stature than 80 per cent of healthy American boys. In the group 10-14 years of age, 3 of 10 boys were underweight and 7 were underheight according to these American standards. Somewhat similar findings were recorded among the girls, as indicated in Fig. 2. It should be noted that none of the children were above the ranges in height or weight. The intercostal diameter was measured in the children aged 3-6 years and was found to be within the range of normal American children as given by Vickers and Stuart.<sup>13</sup>

In an appraisal of general attitude and mental and emotional reactions, it was observed that both women and children were extremely shy and often apathetic. The children, especially, lacked spontaneity and were inactive and quiet. There was no enthusiasm or exuberance of spirit and little inquisitiveness was manifest during the examination procedure. Children appeared older than their years as far as facial expression was concerned. Very little humor was exhibited, and in adults a lack of initiative with an attitude of acceptance of difficulties was the rule. Apathy in the children and the paucity of ambition and "pep" were noted by Mitchell<sup>6</sup> in her studies in Newfoundland. However, the lack of organized play may be a contributing factor to these conditions.

*Laboratory Findings.*—Hemoglobin determinations were made in 37 women and 42 children. In Table V are given the findings as well as the criteria used in judging the degree of anemia. The incidence of anemia, as might be

# HEIGHT

(LENGTH BIRTH TO 3 YEARS)

# BOYS

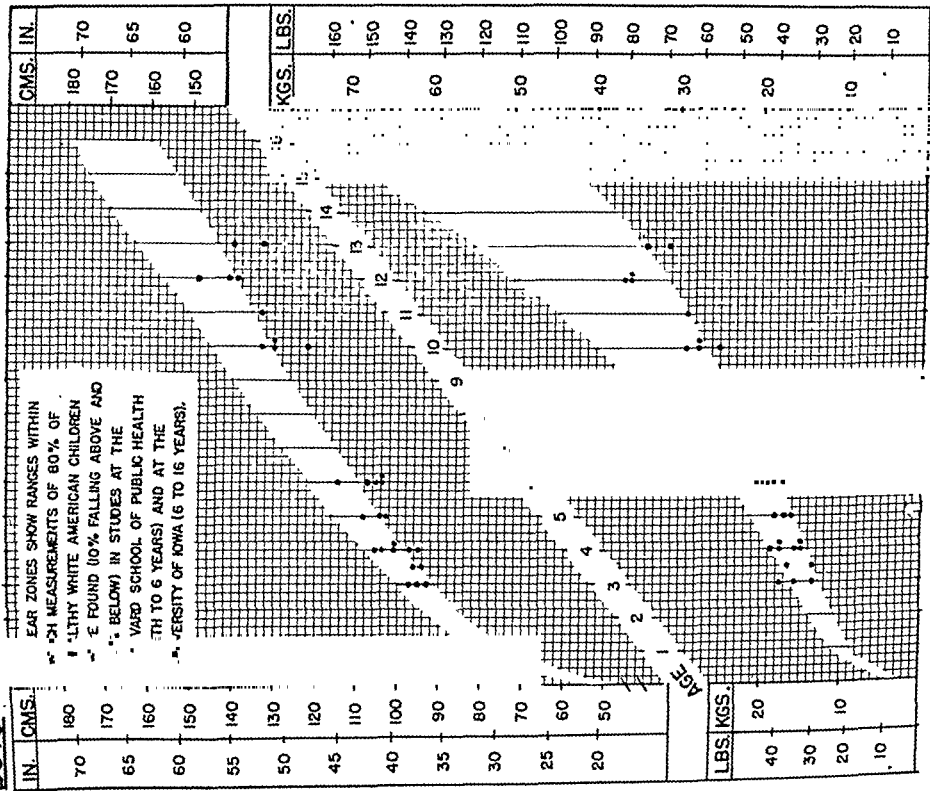
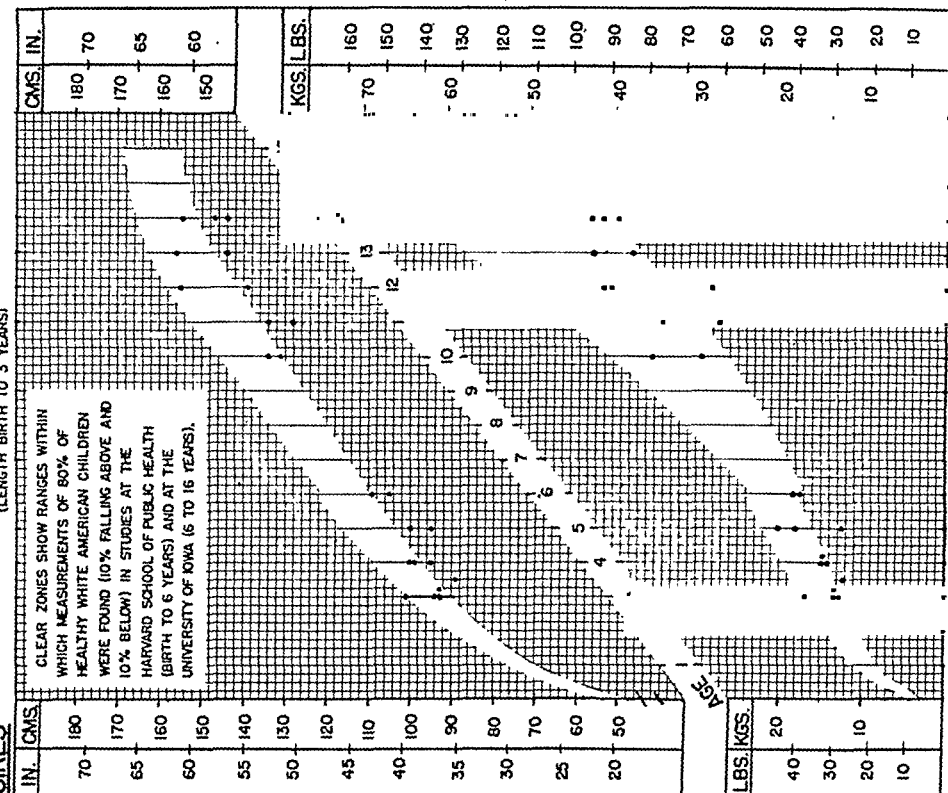


Fig. 1.—Height and weight of Newfoundland boys compared with American boys.

# HEIGHT

(LENGTH BIRTH TO 3 YEARS)

# GIRLS



# WEIGHT

Fig. 2.—Height and weight of Newfoundland girls compared with American girls.

TABLE V. INCIDENCE OF ANEMIA AS INDICATED BY HEMOGLOBIN DETERMINATIONS

	WOMEN (37)			CHILDREN 3-6 YR. (21)			CHILDREN 10-14 YR. (21)		
	HEMO- GLOBIN (GM. %)	NO. OF CASES	PER CENT CASES	HEMO- GLOBIN (GM. %)	NO. OF CASES	PER CENT CASES	HEMO- GLOBIN (GM. %)	NO. OF CASES	PER CENT CASES
Severe anemia	< 10.0	1	2.7	< 9.5	0	0.0	< 10.0	0	0.0
Moderate anemia	10.0-11.5	11	29.7	9.6-10.5	1	5.0	10.1-11.0	2	9.5
Slight anemia	11.6-12.5	10	27.0	10.6-11.5	7	33.0	11.1-12.0	2	9.5
No anemia	> 12.5	15	40.5	> 11.5	13	60.0	> 12.0	17	81.0

anticipated, is highest in the women, 60 per cent of the sample, while 40 per cent of the younger and 19 per cent of the older group of children had subnormal levels of hemoglobin.

Plasma protein determinations were carried out in 38 of the women and 43 of the children. The results are given in Table VI. A protein level of less than 6.0 Gm. per cent was considered suggestive of hypoproteinemia, and this was found in 6 children and 3 women.

TABLE VI. PLASMA PROTEIN VALUES

GROUP	GRAMS PER CENT OF PLASMA PROTEIN					
	<5.5	5.5-5.9	6.0-6.4	6.5-6.9	7.0-7.4	7.5+
Women (38)	0	3	7	18	9	1
Children 3-6 yr. (21)	1	5	9	5	1	0
Children 10-14 yr. (22)	0	0	4	13	5	0

## DISCUSSION

An attempt was made to determine the frequency with which nutritional deficiency occurred in the sample by evaluating symptoms, objective physical signs, and laboratory data. All of the symptoms and signs listed in Tables III, IVA, and IVB and some of the laboratory data in Tables V and VI are suggestive of various types of inadequate nutrition, although none alone is pathognomonic for a nutritional deficiency. They must be interpreted together and evaluated in terms of the individual as a whole. As a convenient and conservative way of applying the data obtained in the diagnosis of deficiency disease, a purely arbitrary classification was set up and each individual was diagnosed as having apparent, suggestive, questionable, or no deficiency in regard to each of the vitamins, as well as to iron and protein. Apparent deficiency was diagnosed if the person showed three signs or two signs and one or more symptoms which are considered to be characteristic of deficiency of a given dietary substance. Suggestive deficiency was indicated when the person showed two signs, or one sign and one or more symptoms. A person was designated as having questionable deficiency if only one sign was present or if signs were less definite. Symptoms alone were not used as the basis for the diagnosis of a deficiency. Individual judgment plays a considerable role in any such classification, and some of the criteria used in the evaluation may be shown to be incorrect. Nevertheless, such an evaluation gives an estimate of the incidence of deficiency disease in the community studied.

The results are given in Table VII, and it is observed that 41 per cent of the total sample were judged to have an apparent deficiency; 37 per cent, suggestive deficiency; 13 per cent, questionable deficiency; and only 9 per cent, no deficiency. The incidence of apparent deficiency was highest in the women, next highest in the men, and lowest in the children of the 3-6-year group.

The incidence of apparent and suggestive deficiency of individual factors is given in Table VIII. Deficiency of vitamin A, riboflavin, and iron were the conditions most frequently encountered. Women showed the highest in-

# BOYS

## HEIGHT

(LENGTH BIRTH TO 3 YEARS)

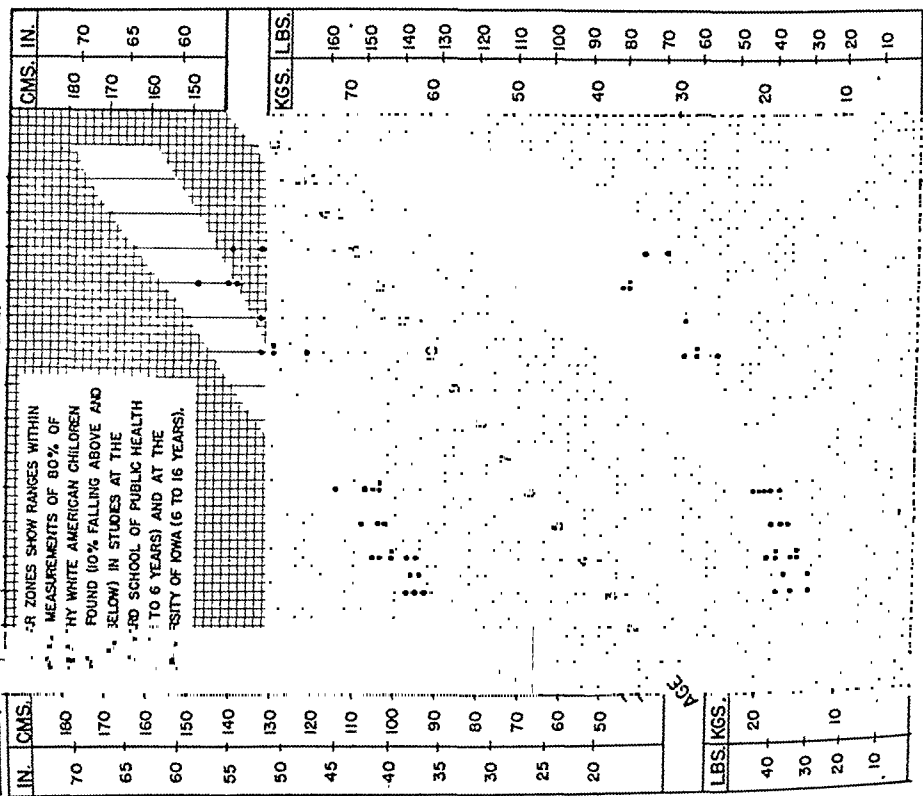


Fig. 1.—Height and weight of Newfoundland boys compared with American boys.

# GIRLS

## HEIGHT

(LENGTH BIRTH TO 3 YEARS)

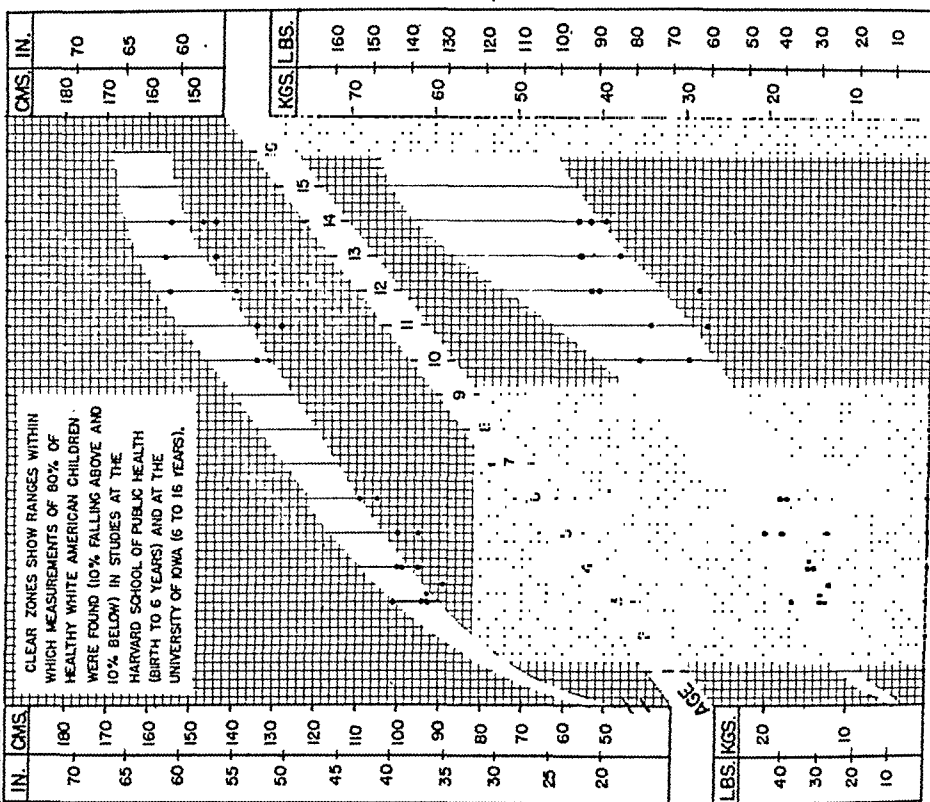


Fig. 2.—Height and weight of Newfoundland girls compared with American girls.



vidence of deficiency of riboflavin and iron, while men showed a greater incidence of vitamin A deficiency. There were no instances of typical pellagra, and niacin deficiency was considered to be infrequent. We felt that in many instances an inadequate supply of niacin accompanied riboflavin deficiency, although signs suggestive of the latter dominated the clinical picture.

Thiamine deficiency as indicated by objective findings was infrequent, but symptoms suggestive of this deficiency were common; however, using the criteria arbitrarily set up, a diagnosis of apparent or suggestive thiamine deficiency could rarely be made. In addition, few men were included in the sample, and it was pointed out by Aykroyd<sup>2</sup> that 80 per cent of the beriberi he observed in Newfoundland occurred in men. The low incidence of ascorbic acid deficiency is undoubtedly due to the rigid criteria used in this survey. If the presence of gum changes alone was considered to be definite evidence of vitamin C deficiency, the incidence would be extremely high. If plasma or whole blood ascorbic acid values could have been determined, they probably would have been low. This would have given another objective sign and the incidence of ascorbic acid deficiency as judged by the arbitrary criteria set up would have increased considerably. Twelve children of the 58 examined had signs of old rickets. Hypoproteinemia was rarely observed except in the young children, and here it may be questioned as to whether a plasma protein value of less than 6.0 Gm. per cent is indicative of hypoproteinemia.

Many persons had a deficiency of more than one essential food factor. The distribution of multiple deficiencies is given in Table IX.

The knowledge that nutritional requirements are elevated during pregnancy and the increasing realization of the importance of good nutrition during this period make it of interest to evaluate these data with reference to the clinical incidence of nutritional deficiency in relation to pregnancy and to the age of the mother. In the sample of 39 mothers, 36 (92 per cent) had either an apparent or suggestive deficiency of any of the several types observed. The incidence of deficiency associated with pregnancy is high and remarkably constant irrespective of the number of pregnancies as shown in Table X.

The most prominent deficiencies in the adult women were vitamin A, riboflavin, and iron. Many mothers had combinations of these deficiencies. The total incidence of these deficiencies correlated with the number of pregnancies and with age of the mothers is given in Table XI. There appears to be an increasing incidence of vitamin A deficiency with additional pregnancies and in the age group over 36. Deficiency of riboflavin and iron was higher in the group with four to six pregnancies than in the group with fewer pregnancies. Deficiencies of these two nutrients were higher in the group 26 to 30 years of age.

TABLE XI. SPECIFIC NUTRITIONAL DEFICIENCIES COMPARED WITH NUMBER OF PREGNANCIES AND AGE

	NUMBER OF WOMEN	VITAMIN A DEFICIENCY*	RIBOFLAVIN DEFICIENCY*	IRON DEFICIENCY*
Number of pregnancies				
1-3	13	4 (31%)	5 (38%)	7 (58%)
4-6	16	11 (69%)	13 (81%)	11 (69%)
7-12	10	9 (90%)	4 (40%)	4 (44%)
Age group (yr.)				
18-25	10	4 (40%)	4 (40%)	5 (50%)
26-30	10	6 (60%)	9 (90%)	7 (70%)
31-36	9	5 (56%)	5 (56%)	5 (63%)
Over 36	10	9 (90%)	4 (40%)	5 (56%)
Total	39†	24 (62%)	22 (56%)	22 (59%)

\*Deficiency—both apparent and suggestive deficiency are grouped in this category.

†Hemoglobin determined in 37 patients.

If one correlates the total number of all the individual deficiencies in the group of women with the number of pregnancies and age, a relationship similar to that of riboflavin and iron is obtained; that is, there was a greater incidence of all deficiencies in the group with four to six pregnancies and in the group 26 to 30 years of age. In connection with these correlations, it should be noted that the number of pregnancies increased with the age of the mother. It follows that the influence of pregnancy alone in increasing the incidence of deficiency disease cannot be ascertained from these data.

An interesting finding was noted when the family groups were studied as a unit for incidence of nutritional deficiency. In a majority of instances the same type of deficiency disease was noted in the children as was shown by the mother. In some families almost identical lesions were seen in each of the members. This was particularly true of changes in the tongue. The incidence of deficiency was highest in the children of mothers who had had five or six pregnancies (79 per cent) and next highest in the children of mothers who had had three or four pregnancies (57 per cent).

From the records of the local hospital it was possible to obtain information regarding the administration of vitamin preparations, liver, and iron to persons included in this survey. Fourteen persons had received iron; 14, some source of the vitamin B complex; 12, cod-liver oil; and 2, thiamine. Of this group of 42 persons who had received therapy, 21 had no evidence of the nutritional deficiency for which the supplements had been prescribed. A number of persons who had received no medication were also without evidence of deficiency disease; for example, 10 women had no anemia and 11 no evidence of riboflavin deficiency. It is probable that the persons treated were those with severe deficiency states and that as a result of therapy, the incidence and severity of nutritional deficiency were somewhat decreased.

A few other observations made during the survey may be mentioned. There was practically no hypertension. One man had a blood pressure of 160/90, while 2 women had blood pressures of 140/105 and 140/100, respectively. The incidence of tuberculosis is high in Norris Point, but only one of the persons examined had evidence of tuberculosis and the lesion was minimal. The death rate from tuberculous meningitis is relatively high in children. Of the women examined, 2 had diffuse and one nodular nontoxic goiter, 2 showed signs of mild hyperthyroidism, and 1 of severe hypothyroidism. Three women had symptoms suggesting chronic cholecystitis, and 1 man had a peptic ulcer. Three women gave a history of some type of toxemia of pregnancy. There were 3 instances of definite and 3 of probable heart disease among the women, 2 of heart disease in the children, and 1 in the group of men. Rheumatic fever is the usual etiologic factor in heart disease in the community of Norris Point.

#### SUMMARY

A survey of the nutritional status of a selected sample of recently pregnant women, their preschool and preadolescent children, and their husbands and a few additional men living in the community of Norris Point, Newfoundland, is reported. The sample was selected to represent the poorest nutrition in the community and consisted of 39 women, 34 children in the age group 3-6 years, 24 children 10-14 years of age, and 16 men. This group comprised about 40 per cent of the entire population of the village.

On the basis of medical histories, physical examination, hemoglobin and plasma protein determinations, the incidence of nutritional deficiency among the women was as follows: apparent, 61 per cent; suggestive, 31 per cent;

questionable or none, 8 per cent. Children were similarly evaluated and showed the following: preschool children—apparent deficiency, 23 per cent; suggestive, 47 per cent; and questionable or none, 30 per cent; preadolescent children—apparent deficiency, 29 per cent; suggestive, 29 per cent; and questionable or none, 42 per cent. Of the men, 44 per cent had apparent, 44 per cent suggestive, and 12 per cent questionable or no deficiency. Multiple deficiencies were the rule, and those most frequently encountered were vitamin A, riboflavin, and iron in the women and children; vitamin A and riboflavin in the men. Dietary histories were taken but were not used in estimating the incidence of deficiency disease because of the inaccuracies inherent in this procedure.

A hemoglobin of less than 11.5 Gm. per cent was found in 32 per cent of the women and in 7 per cent of the children. A plasma protein value of less than 6.0 Gm. per cent was found in 8 per cent of the women and 14 per cent of the children. Marked dental caries was recorded in 44 per cent of the women, 29 per cent of the children, and 31 per cent of the men. Severe periodontal disease was noted in 26 per cent of the women, 3 per cent of the children, and 19 per cent of the men. Twenty per cent of the children had evidence of old rickets.

The findings indicate a need for remedial measures. An isolated community such as Norris Point is an ideal laboratory for investigation of the value of various therapeutic procedures.

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venous use as blood substitutes,\* and two pharmaceutical gelatins, Pharmagel A and Pharmagel B.† Two soybean phosphatide preparations,‡ commercial soybean lecithin and purified soybean phosphatides, were also used. Gum arabic was found unsatisfactory as a stabilizing agent and hence was not studied.

A series of corn oil (Mazola) emulsions was prepared with each stabilizer in which the pH and the concentration of the stabilizer were varied. In each case an attempt was made to find the conditions most favorable for the action of the stabilizer and thereby reduce the necessary quantity to a minimum. When an emulsion of reasonable stability and employing a minimum of stabilizer was obtained, it was used in an infusion experiment. All emulsions were autoclaved at 15 pounds' pressure for twenty-five minutes before use. The composition of seven emulsions stabilized with Igepon T, cetyl phosphoric acid, and the gelatins is given in Table I; that of five emulsions stabilized with soybean phosphatides is given in Table II.

TABLE I. COMPOSITION OF EMULSIONS USED IN EXPERIMENTS 1 THROUGH 9

EMULSION NUMBER	5A	5E	6H	6J	7F	8A	8
<i>Grams Per Liter</i>							
Water	925	930	925	925	881	740	890
Corn oil	67	67	67	67	105	208	89
Haliver oil	2		0.9				0.9
Igepon T							17.8
Cetyl phosphoric acid			2.67	2.67			
Pharmagel A					9.5		
Pharmagel B						13.5	
Gelatin (S. S. Pierce)	6.0						
Knox gelatin P6-00		9.4					
NaCl	1.89	1.89	1.87	1.87			3.89
KCl	1.85		1.85				2.47
Na <sub>2</sub> HPO <sub>4</sub>	2.26	3.02					1.37
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O		1.96	0.72	0.38	0.07		0.15
Tartaric acid					0.8		
NaHCO <sub>3</sub>						4.5	
NaOH			2.0	2.0	0.6		
pH	7.6	6.9	7.4	7.4	8.1	8.5	7.4

TABLE II. COMPOSITION OF EMULSIONS USED IN EXPERIMENTS 10 THROUGH 16

EMULSION NUMBER	10B	10D	11H	11J	11M
<i>Grams Per Liter</i>					
Water	870	870	800	800	800
Corn oil	100	100	150	150	
Coconut oil					150
Commercial soybean lecithin	30	20			
Purified soybean phosphatides			36	18	27
Na <sub>2</sub> HPO <sub>4</sub>	4.14	2.76	8.0	4.0	6.0
pH	7.25	7.25	7.6	7.6	7.6

*Infusion Experiments.*—Normal adult dogs were used in all of the experiments, and they were maintained on a Purina dog chow ration fed ad libitum. In all experiments except 1 and 2 they were trained to lie down on an animal table, and the fat emulsion was given from a Murphy drip bottle into a leg vein in the usual fashion. In the first two experiments the dogs were subjected to continuous infusion from an apparatus similar to that described by Jacobs.<sup>11</sup> All emulsions not prepared isotonic were rendered so at the time of infusion by the addition of suitable quantities of sodium chloride solution. Infusion Experiments 1 through 9 are summarized in Table III; Experiments 10 through 16, in Table IV.

\*Knox Gelatin Co., Johnstown, N. Y., and Upjohn Co., Kalamazoo, Mich.

†Pharmagel Corporation, New York, N. Y.

‡American Lecithin Company, Elmhurst, Long Island, N. Y.

## DISCUSSION

From the results given in Table III, it can be seen that all of the emulsions used in Experiments 1 to 9 inclusive were highly toxic. Infusion of emulsion 8, which was stabilized with Igepon T, resulted in death of the animal (Experiment 1) in three days, although infused very slowly. The cause of death was in all probability the result of extensive pulmonary infarction. The vomiting and excessive salivation observed also indicated toxicity, perhaps due to the surface-active stabilizer. These symptoms were noted when emulsions stabilized with Igepon T were infused into dogs in our earlier studies employing the hand homogenizer. Emulsions stabilized by cetyl phosphoric acid produced fatal pulmonary embolism in Experiments 4 and 6 (emulsions 6H and 6J). In Experiment 3, emulsion 6H produced thrombophlebitis of such extent that the experiment had to be terminated. In Experiment 6 an infusion of 95 c.c. produced fatal consequences on the first day of the experiment. Emulsions 6H and 6J were markedly hemolytic in vitro. It is interesting that comparable quantities of cetyl phosphoric acid dispersed in water do not produce such effects when injected intravenously. Evidently much of the toxic effect of this substance depends on its orientation at an oil-water interphase. The high toxicity of these emulsions is interesting in view of the fact that, owing to its efficiency as a stabilizer, relatively small quantities of cetyl phosphoric acid were used in their preparation.

Emulsions stabilized with the various gelatin preparations caused pulmonary embolism (Experiments 2, 5, 7, 8, 9, Table III). There was little variation in the pattern of symptoms. Labored abdominal breathing, salivation, vomiting, and defecation occur in rapid succession within fifteen minutes to one hour after beginning the infusion. If the infusion is not stopped at this stage, death may occur. The gelatins are well tolerated when infused alone in a solution of

TABLE III. SUMMARY OF INFUSION EXPERIMENTS 1 THROUGH 9

EXPERIMENT	EMULSION	STABILIZER	WEIGHT OF DOG (KG.)	SEX	DURATION OF EXPERIMENT (DAYS)	AVERAGE DAILY AMOUNT USED (C.C.)	AVERAGE NUMBER CALORIES PER KG. FROM FAT PER INFUSION	AVERAGE INFUSION TIME (MIN.)	GROSS EFFECTS OF INFUSION
1	8	Igepon T	13.8	F	3	450	26	880	Vomiting; excessive salivation; death
2	5A	Gelatin (Overland)	12.3	F	2	500	24.5	950	Vomiting; salivation; convulsions; pulmonary embolism; death
3	6H	Cetyl phosphoric acid	13.6	M	2	118	5	120	Experiment stopped—all veins thrombosed
4	6H	Cetyl phosphoric acid	16.2	M	2	137	5		Pulmonary embolism; death
5	5E	Gelatin (Knox) PG-00	10.3	M	3	232	13.5	101	Coughing, abdominal respiration, and defecation during all infusions—stopped experiment
6	6J	Cetyl phosphoric acid	12.5	M	1	95	4.5	65	Vomiting; defecation; urination; pulmonary embolism; death
7	8A	Pharmagel B		F	1	25		15	Vomiting; defecation; and labored breathing—stopped experiment
8	7F	Pharmagel A		F	1	64		22	Vomiting; defecation; urination; salivation; labored breathing—stopped experiment
9	5A	Gelatin (Overland)		F	1	60		20	Vomiting; labored breathing—stopped experiment

the same pH and in quantities considerably in excess of that used in the emulsions. Indeed, the Knox gelatin P6-00 used in emulsion 5E is actually prepared as an infusion colloid. These emulsions all showed a tendency to aggregation into clusters of droplets when mixed with plasma *in vitro*; this undoubtedly accounts for the pulmonary disturbances following infusion. The gelatin-stabilized emulsions demonstrate that toxic emulsions may be prepared from relatively nontoxic stabilizers and oils.

Pharmagel A and Pharmagel B are prepared from acid- and alkali-treated precursors, respectively, and have isoelectric points in the pH ranges 7 to 8 and 4.7 to 5.0.<sup>12</sup> It follows that emulsion 7F stabilized with Pharmagel A at pH 8.1 must have a gelatin film about isoelectric or perhaps slightly negatively charged. The Pharmagel B stabilized emulsion (8A) must have a negatively charged gelatin film. Since plasma proteins are negatively charged at blood pH, there would appear to be no charge incompatibility between these emulsion films and the plasma proteins. Electrophoretic evidence indicates that the chylomicron emulsion particles are negatively charged.<sup>5</sup> The behavior of these emulsions is therefore probably not associated with this factor.

The toxicity of emulsions prepared with these stabilizers induced us to return to commercial soybean lecithin which had been used in our early experiments.<sup>1</sup> It was found that such emulsions were much more stable when prepared with the high pressure homogenizer than with the hand homogenizer. Infusion of emulsion 10B (Experiment 10, Table IV) was much better tolerated, although a transient urticaria developed during the infusion on the sixth day and was much more marked during the infusion on the seventh day. The urticaria involved the skin of the head, causing a general swelling around the face, and also involved the skin of the abdomen. There was considerable hemolysis attending the infusion of this emulsion. Emulsion 10D contained less of the soybean lecithin but likewise produced an urticaria on the first day when infused in another dog (Experiment 11, Table IV). The use of the commercial soybean lecithin as stabilizer was then discontinued. At this time, a purified preparation of soybean phosphatides was made available to us.<sup>\*</sup> This preparation consists of about equal parts of soybean lecithin, cephalin, and lipositol. It is relatively free of soybean oil and free fatty acids. It is a straw-colored stable powder which is readily soluble in water and is a fairly efficient stabilizer above pH 7.2.

Emulsions 11H, 11J, and 11M were prepared, using these purified phosphatides as stabilizer and used in Experiments 12 to 16 inclusive. These emulsions were much better tolerated than any used previously and were therefore infused for longer periods of time. The dogs used in these experiments were sacrificed with nembutal on the day following the last fat infusion. Terminal findings for these dogs are given in Table V.

Experiment 12 was of the longest duration (forty-eight infusions) and throughout the experiment a total of 1.35 kg. of corn oil was infused into the dog. A progressive loss of weight and slight anemia attested to the mild toxicity of this emulsion (11H). However, the animal seemed normal in other respects. Liver function as judged by bromsulfalein elimination, plasma phosphatase, plasma cholesterol components, and plasma protein concentration appeared to be normal (Table V). The methods employed in measuring liver function have been described elsewhere.<sup>13</sup> It is to be noted that the bromsulfalein elimination in this animal is not as efficient as that of the control puppies in the choline deficiency studies,<sup>13</sup> but in all of the dogs of this series it was far superior

\*We are indebted to Dr. Albert Scharf, of the American Lecithin Co., Elmhurst, Long Island, N. Y., for this and other soybean phosphatide preparations.

TABLE IV. SUMMARY OF INFUSION EXPERIMENTS 10 THROUGH 16

EXPERIMENT	EMULSION	STABILIZER	SEX	DURATION OF EXPERIMENT (DAYS)	TOTAL NUMBER OF INFUSIONS	AVERAGE DAILY AMOUNT USED (C.C.)	AVERAGE NUMBER CALORIES PER KG. FROM FAT PER INFUSION	AVERAGE INFUSION TIME (MIN.)	INITIAL WEIGHT (KG.)	FINAL WEIGHT (KG.)	INITIAL HEMATOCRIT (%)	FINAL HEMATOCRIT (%)	INITIAL HB. (GM. %)	FINAL HB. (GM. %)	GROSS EFFECTS OF INFUSIONS
10	10B	Commercial soybean lecithin	F	8	8	198	10.5	126	16.7	16.7	42.0	37.5	13.3	11.3	Urticaria developed by sixth day; occasional shivering; rapid breathing
11	10D	Commercial soybean lecithin		1	1	58	2.5	30	20.7						Immediate urticaria
12	11H	Purified soybean phosphatides	F	55	48		13.6	130	19.5	17.0	37.0	32.5		9.9	Loss of weight; mild anemia
13	11H	Purified soybean phosphatides	M	29	27	200	16.0	141	17.7	16.55	48.0	34.0	14.6	10.5	Loss of weight; mild anemia
13a			M	39	0				16.55	18.1	34.0	42.0	10.5	13.3	Normal after rest period
14	11J	Purified soybean phosphatides	F	20	28	167	16.5	125	13.5	13.85	32.5	29.8	12.1	10.5	None
15	11M	Purified soybean phosphatides	F	20	28	196	16.2	147	15.95	16.75	37.9	36.7	13.3	12.3	None
16	11M	Purified soybean phosphatides	M	30	29	202	15.6	153	15.75	19.2	30.5	36.1	12.1	11.3	None

TABLE V. TERMINAL FINDINGS IN DOGS OF EXPERIMENTS 12 TO 16 INCLUSIVE

	EXPERIMENT				
	12	13	14	15	16
Bromsulphalein test ( $\mu$ g dye per c.c. plasma in 8 minutes)	12	10	11	16	7
Plasma phosphatase ( $\mu$ g phosphorus liberated per c.c. plasma in 24 hr.)	205	409	148	45	57
Plasma "total" cholesterol (mg. per cent)	152	153	256	200	200
Plasma esterified cholesterol (mg. per cent)	114	128	157	138	108
Plasma Proteins (Gm. per cent)	5.3		4.7	5.2	5.35
Total liver lipids (per cent dry weight)	18.9	12.8	14.7	18.8	15.6
Total lung lipids (per cent dry weight)	17.1		19.0	16.9	16.8
Total kidney lipids (per cent dry weight)	16.2			19.5	16.8
Total spleen lipids (per cent dry weight)	16.5		11.1	18.0	9.2
Total pancreas lipids (per cent dry weight)			39.0	24.7	22.5

to that of the choline-deficient puppies. Post-mortem examination showed no gross abnormalities with the exception of numerous old renal infarcts. Microscopically, stainable fat was present in phagocytes in the lungs and in liver parenchymal and Kupffer cells. In these organs and in the kidneys a cellular reaction to this stored foreign lipid had evidently occurred, giving rise to scattered, discrete, occasionally partially necrotic granulomatous lesions. These

consisted of central lipid-laden macrophages and occasional giant cells with surrounding collars of lymphocytes. In addition there was evidence of moderate but widespread deposition of blood pigment in reticulo-endothelial phagocytes.

The visceral organs showed normal lipid contents as measured by chloroform extraction. Infused fat does not, therefore, accumulate in these organs. We have found that it is not excreted in the urine. The fate of the mass of corn oil infused in emulsion form is a question of considerable importance. It appears doubtful that any quantity would be excreted by way of the gastrointestinal tract. The conclusion seems inescapable that (1) it is deposited in the fat depots and/or (2) it is burned for energy. Careful gross observation made it doubtful that the dog in Experiment 12 had 1.35 kg. of total body fat. It seems likely, therefore, that the infused fat was utilized for energy. This conclusion is in harmony with those reached from respiratory quotient studies following infusion of fat emulsions into dogs,<sup>14</sup> depancreatized dogs and rabbits,<sup>15</sup> and human infants.<sup>16</sup> It is not in agreement with the conclusions of Dunham and Brunschwig<sup>3</sup> who failed to observe (1) consistent changes in the character of depot fat toward that of the infused fat, and (2) reduction of nitrogen excretion on low caloric intakes during fat infusion periods. The first criterion of utilization is a precarious one; the second would not be expected to be valid during active hemolysis as was the case in their dogs. To us the conclusions of these investigators seem unwarranted. For the present, however, evidence for utilization of infused fat is not unequivocal. Further studies on this question are now in progress.

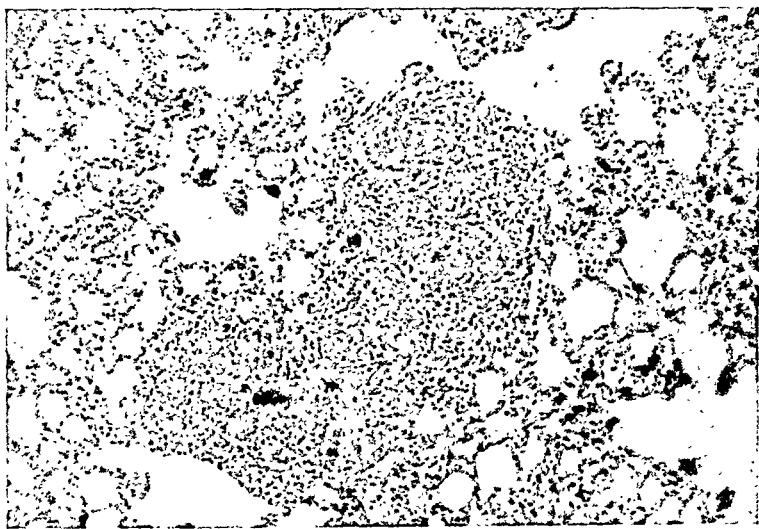


Fig. 1.—Photomicrograph of lung section (Dog of Experiment 16) showing noncaseating granulomatous lesion. Note the giant cell (hematoxylin and eosin  $\times 100$ ).

In Experiment 13, emulsion 11H produced a slight but significant loss in weight and a reduction in hemoglobin and hematocrit over a shorter period of time. This dog was not sacrificed until thirty-nine days after the infusions were stopped (Experiment 13a, Table IV). During this interim, its weight and blood picture returned to normal. Post-mortem studies revealed lesions similar to, but not as extensive as, those seen in the previous experiment. It is to be noted, however, that this dog received far fewer infusions than did the animal of Experiment 12.



In an attempt to reduce the toxicity of emulsion 11H, two other emulsions were prepared. Emulsion 11J (Table II) contained only half as much stabilizer as emulsion 11H and was used in Experiment 14 (Table IV). This animal showed a slight gain in weight after twenty-eight infusions and appeared to be tolerating the infusions better than the dogs receiving emulsion 11H. Terminal liver function and other findings were normal (Table V). It is apparent that part of the untoward effects are due to an excess of the phosphatides. The effects of the oil are not to be overlooked. Koehne and Mendel<sup>17</sup> found marked differences in the utilization of different oils given parenterally. Coconut oil was found to be superior to cod-liver oil, butter, and peanut oil in this respect. Accordingly, another emulsion, 11M, was prepared which contained coconut oil\* instead of corn oil (Table II). This emulsion was used in Experiments 15 and 16. Both of the animals in these experiments tolerated the emulsion very well and appeared normal after twenty-eight and twenty-nine daily infusions. Particularly interesting was Experiment 16 in which an undernourished dog was brought back to normal during the infusion period. Its net gain was 3.45 kg., representing a gain of 21.9 per cent of the original body weight. The terminal liver function and the lipid contents of visceral organs of both dogs receiving emulsion 11M were normal (Table V). However, in the lungs of all of these animals phagocytosis of fat and a noncoagulating cellular response to the foreign lipid were evident (Fig. 1).

## COMMENT

These experiments clearly demonstrate that the stabilizer is a prime factor in determining the tolerance of an animal to an emulsion. All of the emulsions used in Experiments 1 to 14 inclusive were emulsions of corn oil with comparable particle size and pH. The differences in tolerance must have resulted from differences in the stabilizers employed. Of the series of stabilizers we have used, only the soybean phosphatides stand out as suitable for this purpose. If an oil-in-water emulsion is to be used as a carrier for fat-soluble vitamins, or drugs, or to absorb toxins or for any other purpose where relatively small amounts are needed, a number of other stabilizers may be satisfactory. However, for the purpose of introducing enough fat to meet requirements for parenteral nutrition, a toxicity restriction is imposed on many stabilizers and their emulsions. The literature on this subject gives but few accounts of fatal outcome following the infusion of emulsions. Perhaps the chief reason is that very small quantities have been used in most of the experiments. Dunham and Brunschwig<sup>3</sup> state that nine of twenty-four dogs used in their studies died because of the infusions. Their emulsions were stabilized with egg phosphatides and "Demal." McKibbin and co-workers<sup>1</sup> reported fatal outcomes in preliminary experiments on complete parenteral nutrition, but this cannot be attributed simply to the soybean phosphatides or their emulsions since other factors may have operated in experiments of this kind. As far as we are aware, no other investigators have reported fatal terminations of fat emulsion infusion experiments. Whether or not egg lecithin or egg phosphatide preparations may be toxic under certain conditions is an important point raised by the studies of Dunham and Brunschwig. One might expect egg and soybean phosphatides to have much in common, although there are rather marked differences in their composition. The egg phosphatides contain lecithin (2 molecules of beta to 1 of alpha), cephalin, and usually considerable cholesterol. The soybean phosphatides consist of lecithin (2 molecules of alpha to 1 of beta), cephalin, and lipositol. What

\*Refined coconut oil, E. F. Drew & Co. Inc., Boonton, N. J.

differences, if any, are inherent in the lecithins other than their alpha to beta ratios, in the cephalins, in contaminating impurities, and in the general physiologic properties of their mixture are obscure. It is of interest that we have had no fatalities in a total of eleven dogs receiving emulsions stabilized with the soybean phosphatides. However, smaller amounts of soybean phosphatides were used in our emulsions than the egg phosphatides used by Dunham and Brunschwig in their emulsions. As to relative efficiency as a stabilizer, little can be said at present. Emulsions 11H, 11J, and 11M all show small but visible droplets of oil at the surface after autoclaving. The small droplets tend to float on the liquid surface in the infusion bottle and gather in the last few cubic centimeters of emulsion where they may be discarded. Hence the larger droplets are not infused. The emulsions are probably not stable indefinitely. In all of our experiments we have used emulsions made up every two weeks. Under these conditions, we have observed no outward signs or symptoms of fat embolism. It is possible that the emulsions could be used after much longer times. More work needs to be done before the relative usefulness of the egg and soybean phosphatides in this capacity can be determined. From the standpoint of availability, general stability, and ease in handling, the advantage thus far seems to lie in the direction of the soybean phosphatides.

Coconut oil appears to be superior to corn oil as an infusion fat. It is relatively nonirritating and when its emulsion is infused subcutaneously (when the needle works out of the vein, as happens occasionally), there is no local tissue reaction as is the case with the corn oil emulsions. This is similar to the findings of Koehne and Mendel.<sup>17</sup> With corn oil this reaction may produce swelling and soreness of the entire area, rendering the leg useless for further infusion for a week or more. One instance of peripheral neuritis appeared to result from such an accident. This constitutes a practical advantage in the use of coconut oil. A number of properties of coconut oil may be desirable for parenteral nutrition mixtures and may make it superior to many other common oils. It is almost entirely saturated, having an iodine number of 6 to 10, and would thus be much less susceptible to oxidative rancidity than other oils. It has a greater proportion of low molecular weight fatty acids (very high saponification number) and soluble fatty acids (relatively low Hehner number). These factors would be expected to increase the rate of utilization of infused fat. Experimental studies designed to evaluate the various oils or fats as to their relative suitability for this purpose are needed.

The histologic examinations reported in these studies show that even the best emulsions (11J and 11M) were not entirely inert over the time they were infused. The available evidence suggests that the foreign lipid is phagocytized by macrophages chiefly in the lungs and then gives rise to a cellular reaction resulting in the formation of noncaseating submiliary granulomatous lesions not unlike tubercles. The sequence of the changes producing these lesions and their subsequent fate cannot be determined with the material at present available for study. Lesions of this kind have not been reported by other investigators following infusion of fat emulsions.

The total energy content of all of the emulsions stabilized with the purified soybean phosphatides (11H, 11J, and 11M) was approximately 1,350 calories per liter on the basis of the fat alone. The energy furnished by the phosphatides themselves, if any, would augment this figure. On the basis of 1,350 calories per liter, it would be possible to give an "average fat intake" to a man weighing 70 kg. (750 calories or 25 per cent of the total caloric intake) with an infusion

volume of 555 c.c. It would be possible to increase the fat concentration of the emulsions should this be desirable.

## SUMMARY

Corn oil emulsions of fine particle size have been prepared with a number of different stabilizers. These have included Igepon T, cetyl phosphoric acid, edible gelatin, pharmaceutical gelatins, infusion gelatins, commercial soybean phosphatides, and purified soybean phosphatides. Emulsions prepared with all these stabilizers except the last two produced severe or fatal reactions when infused into dogs in amounts sufficient to equal the usual caloric intake of fat or less. Emulsions stabilized with the commercial soybean phosphatides produced hemolysis and urticaria, whereas those stabilized with the purified soybean phosphatides produced only minor changes and these after relatively long periods of daily infusions. Refined coconut oil emulsions stabilized with the purified soybean phosphatides have so far been the more successful in infusion studies.

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## TIMING OF PHAGOCYTTIC CHANGES IN MALNUTRITION

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**I**N OUR previous reports<sup>1, 2</sup> on phagocytic changes in the malnutrition of vitamin or protein deficiency, we stated that the animals were kept on the deficient diets from four to seven weeks before being tested for phagocytic efficiency. Preliminary observations had indicated four weeks as the minimal time for full effects of the malnutrition to develop. We wish now to report a more detailed study of the time relationships involved.

Weanling white rats (Sprague-Dawley males) were placed in tropical warmth (90 to 91° F. and 60 to 70 per cent relative humidity) and kept on synthetic diets for eight months before being used for the study. The rats in one group received the optimal diet for tropical warmth described previously,<sup>1</sup> while those in the other group received a diet moderately deficient in protein and all the B vitamins (Table I).

While this low vitamin rat diet would appear to be only mildly deficient, it was about as low as would be tolerated by eight-month-old rats. Further reduction of thiamine from 1.2 mg. per kilogram down to 1.0 mg. per kilogram resulted in typical severe deficiency symptoms and death within four to five weeks. Rats of this age, kept since weanlings in the heat on the optimal hot room diet, also develop fatal thiamine deficiency in about the same time if the dietary thiamine is reduced to 1.0 mg. per kilogram.

Using the technique previously described, we measured the phagocytic activity of the blood polymorphonuclear neutrophils and then placed the rats with deficiencies on the optimal diet while changing some of the normal rats to the deficient diet. Estimates of phagocytic activity and weight change were made weekly thereafter for each group. Four rats in each group were bled from the heart and discarded from the study at the end of each week so that the study would not be complicated by any possible effect on phagocytosis from repeated bleedings.

In Table II is set forth the changes in phagocytic activity and body weight which took place from week to week. Even though the dietary shifts in each case were promptly reflected in body weight changes within the first week, no corresponding alterations were found in activity of the blood phagocytes. By the end of the second week a moderate change was observed in phagocytic function; this became more marked during the third week and was complete by the end of the fourth week (Fig. 1). After four weeks, the rats that formerly had deficiencies exhibited normal phagocytic activity, while those previously normal were now at the low level of full deficiency.

With bacterial counts made in 40 phagocytes from each rat (making 160 cells for each mean value recorded in the table), differences in one day's readings greater than 1.5 become statistically significant. Naturally the readings of one week can be compared with those of another week only by reference to each week's normal values obtained on the control rats. The bacterial suspension used in the third week's tests was slightly too dilute, while that of the fourth week was distinctly heavy. This necessitated the calculation of

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TABLE I  
AMOUNTS OF B VITAMINS AND CASEIN PER KILOGRAM OF DIET

	OPTIMAL DIET FOR TROPICAL WARMTH	DEFICIENT DIET
Thiamine hydrochloride	2.5 mg.	1.2 mg.
Riboflavin	4.0 mg.	1.5 mg.
Pyridoxine	4.0 mg.	1.5 mg.
Calcium pantothenate	6.0 mg.	1.5 mg.
Nicotinic acid	25.0 mg.	10.0 mg.
Choline chloride	5.0 Gm.	1.0 Gm.
Inositol	1.0 Gm.	0.4 Gm.
P-aminobenzoic acid	0.3 Gm.	0.1 Gm.
Casein, vitamin-free	180.0 Gm.	120.0 Gm.

TABLE II  
WEEKLY CHANGES IN PHAGOCYtic ACTIVITY

CONTROL RATS ON FULL DIET (MEAN NUMBER OF BACTERIA PER CELL)	TIME ON NEW DIET (WEEKS)	RATS CHANGED FROM FULL TO DEFICIENT DIET		RATS CHANGED FROM DEFICIENT TO FULL DIET	
		MEAN NUMBER OF BACTERIA PER CELL	PER CENT OF NORMAL	MEAN NUMBER OF BACTERIA PER CELL	PER CENT OF NORMAL
6.12 $\pm$ 0.36	1	6.59 $\pm$ 0.33	108	2.65 $\pm$ 0.18	43
6.17 $\pm$ 0.37	2	5.04 $\pm$ 0.27	82	4.10 $\pm$ 0.31	66
5.37 $\pm$ 0.33	3	2.51 $\pm$ 0.20	47	5.51 $\pm$ 0.28	103
7.57 $\pm$ 0.37	4	2.87 $\pm$ 0.19	38	8.31 $\pm$ 0.29	110
Weight changes first week		- 10 Gm.		+ 17 Gm.	
+ 16 Gm. 4 weeks		- 68 Gm.		+ 61 Gm.	
340 Gm. Initial average weight		346 Gm.		369 Gm.	

comparative changes on a percentage basis, using the normal controls as 100 per cent in every case. Differences of  $\pm 10$  per cent are of no definite significance here.

One review journal has recently<sup>3</sup> criticised our use of only 40 cells per rat (four rats to each group) as the basis for calculating mean ingestion rates, referring to the custom of counting ingestion in from 200 to 500 cells in human phagocytic studies. To test the stability of our ingestion values, we selected two representative groups from our previous report,<sup>1</sup> Groups 1 and 3 of the riboflavin series in the cold room. In addition to the mean values calculated on counts from 160 cells per group (40 per rat), we recalculated the data on the basis of including only the first 20, 10, 5, and 2 phagocytes seen on each slide. In Table III is set forth the results of this recalculation, and it is clearly indicated that the observed differences in mean ingestion counts maintain their statistical significance when as few as only the first 10 cells per rat are included in the calculation. It may thus safely be accepted as true that counts made on 40 phagocytes per animal, with four animals to each group,

TABLE III

NUMBER OF CELLS COUNTED		GROUP 1		GROUP 3		GROUP DIFFERENCE IN MEANS	SIZE OF DIFFER- ENCE IN MEANS NEEDED FOR SIG- NIFICANCE (P.E. $\times 4$ )
PER RAT	PER GROUP	MEAN NUMBER BACTERIA INGESTED PER CELL	STANDARD DEVIATION OF MEAN	MEAN NUMBER BACTERIA INGESTED PER CELL	STANDARD DEVIATION OF MEAN		
40	160	5.76 $\pm$ 0.27	5.12 $\pm$ 0.19	9.37 $\pm$ 0.28	5.28 $\pm$ 0.20	3.61 $\pm$ 0.39	1.56
20	80	5.23 $\pm$ 0.38	5.03 $\pm$ 0.27	8.88 $\pm$ 0.37	4.97 $\pm$ 0.26	3.65 $\pm$ 0.53	2.12
10	40	4.55 $\pm$ 0.52	4.92 $\pm$ 0.37	8.10 $\pm$ 0.51	4.77 $\pm$ 0.36	3.55 $\pm$ 0.73	2.92
5	20	6.20 $\pm$ 0.86	5.72 $\pm$ 0.61	7.90 $\pm$ 0.85	5.63 $\pm$ 0.60	1.70 $\pm$ 1.21	4.84
2	8	6.23 $\pm$ 1.46	6.12 $\pm$ 1.03	6.73 $\pm$ 1.14	4.79 $\pm$ 0.81	0.50 $\pm$ 1.85	7.40

allow an ample margin of stability. In human case studies, 100-cell counts would provide sufficient stability if the technical details of the method were carefully standardized.

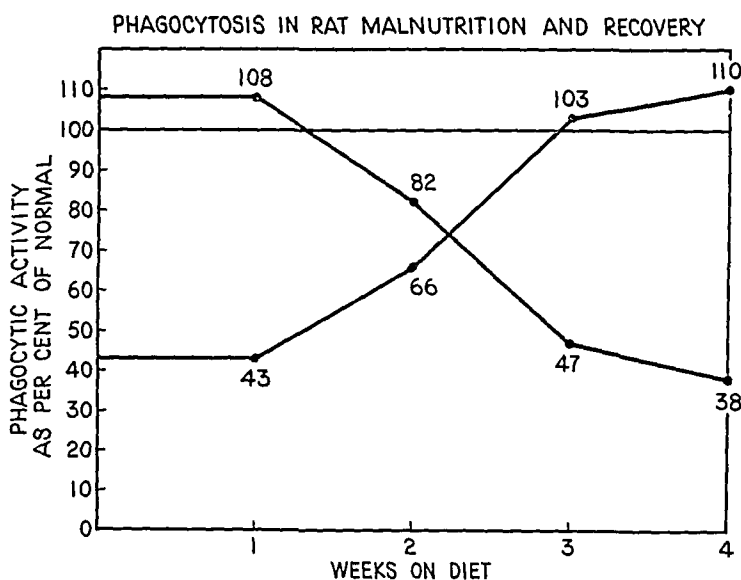


Fig. 1.

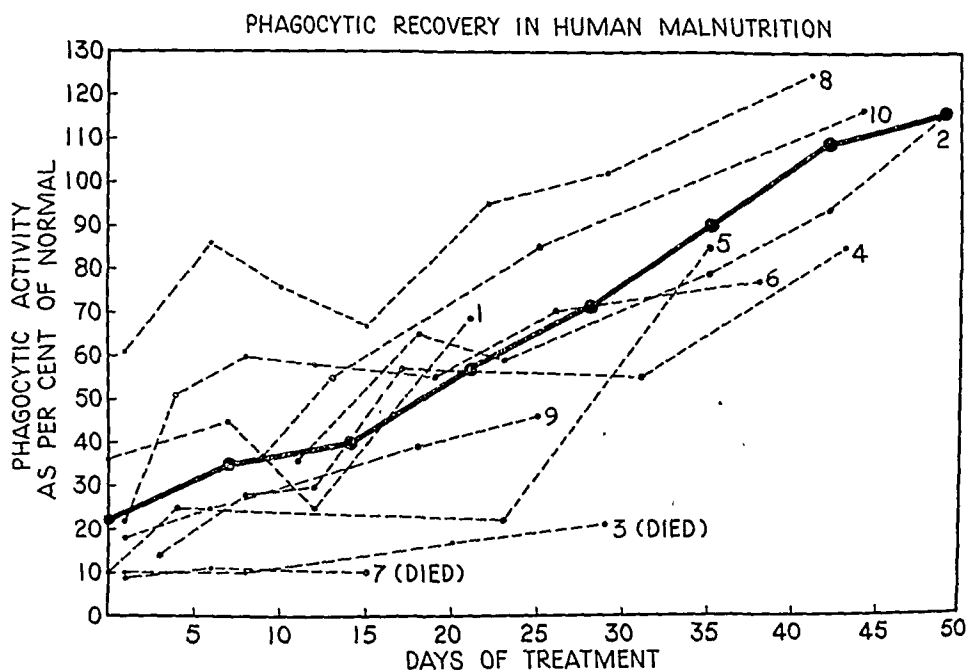


Fig. 2.

#### PHAGOCYTOSIS IN HUMAN MALNUTRITION

We wish also to report preliminary phagocytic studies in a few selected cases of human malnutrition. Several aged patients suffering from bacillary dysentery of variable duration were admitted to the Cincinnati General Hospital from a low-cost private nursing home. All were severely emaciated. The essential features of these cases are presented in Table IV, along with

TABLE IV  
HUMAN CASES OF MALNUTRITION STUDIED

CASE	HOSPITAL NUMBER	SEX	COLOR	AGE (YR.)	DIAGNOSIS	DIET	THERAPY VITAMINS
1	162292	F	W	63	Nicotinic acid deficiency; arteriosclerosis	Regular	Nicotinamide
2	185743	M	W	36	Acute pellagra; chronic alcoholism; peripheral neuritis	Regular	Nicotinamide, thiamine, yeast
3	176787	M	C	10	Miliary tuberculosis; resection of jejunum (died)	Various	Nicotinamide, thiamine, ascorbic acid, CLO
4	175791	F	W	86	Acute enterocolitis, paratyphoid B; avitaminosis; senility	High protein High caloric, soft	Thiamine, riboflavin, nicotinamide, ascorbic acid
5	169544	F	W	80	Acute bacillary dysentery	High protein High caloric, soft	None
6	166581	M	W	73	Acute bacillary dysentery; senility	High protein High caloric, soft	None
7	45506	M	W	76	Acute bacillary dysentery; B vitamin deficiency (died)	High protein High caloric, soft	Thiamine, nicotinamide, ascorbic acid
8	182754	M	W	71	Acute bacillary dysentery; vitamin B deficiency	High protein High caloric, soft	Thiamine, nicotinamide, riboflavin, ascorbic acid
9	186064	M	W	68	Acute bacillary dysentery; malnutrition	High protein High caloric, soft	Thiamine, nicotinamide, riboflavin, ascorbic acid
10	160549	M	W	33	Beriberi heart disease	Regular	Thiamine, yeast, nicotinamide

those of three ward cases of vitamin deficiency and one emaciated patient in whom jejunal resection had been performed (miliary tuberculosis found at autopsy). All the patients with dysentery received succinyl sulfathiazole in adequate dosage, in addition to the special diet.

Phagocytic activity in the blood of these patients was estimated by comparison with two healthy normals as controls, and these normal individuals were at different times checked against healthy rats kept on an adequate synthetic diet to assure constancy.

The average rate of phagocyte recovery in this series of patients with malnutrition was slow during the first two weeks in the hospital, while from the third week onward it was more rapid and remarkably regular (heavy line in Fig 2). It is impossible to say how much of this recovery in the patients with dysentery was due to the succinyl sulfathiazole, how much to the rich diet, and how much to the vitamin therapy. The two patients receiving no vitamin supplementation made about average recovery in phagocytic function. In the first three patients with relatively uncomplicated vitamin deficiencies, good recovery of phagocytic function followed specific vitamin therapy as the only treatment administered. It is perhaps significant that the two patients who died showed very low phagocytic function at all times.

#### DISCUSSION

The general recovery rate in the human patients was somewhat slower than in the rats on synthetic diets, perhaps partly because the rats were other-

wise healthy, while other abnormalities retarded recovery in the human patients. One significant point of the study is the demonstration that malnutrition, from whatever cause, can produce a very marked lowering in phagocytic function. A second point of importance is the finding that phagocytic recovery follows restoration of nutritional adequacy in both rats and men, but with a lag of from one to two weeks.

Presence of this definite lag in phagocytic recovery might possibly be interpreted as meaning that improved phagocytic function comes only with release from the bone marrow of new phagocytes produced under the improved nutritional conditions prevailing. In similar manner, the lag in the decline of phagocytic activity with dietary inadequacy may mean that only new cells being produced under the deficient marrow conditions will show faulty function. Mature phagocytes already in the circulating blood seem to be less affected by the change in nutritional status of the individual.

#### CONCLUSIONS

Rats changed from an adequate to a deficient diet show marked weight loss during even the first week, but reduction in activity of the blood phagocytes becomes evident only during the second week and is completed by the end of the fourth week. Those changed from a deficient to an adequate diet show a similar timing in the recovery of phagocytic activity.

In human malnutrition phagocytic function is low. Dietary correction and vitamin supplementation result in phagocytic improvement which (on the average) follows a straight line rise from the third week onward, reaching normal levels in about five weeks.

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## THE ADMINISTRATION OF PENICILLIN BY CONTINUOUS INTRAMUSCULAR DRIP

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WHEN penicillin is injected parenterally, it is rapidly removed from the blood, so that concentrations remaining in the serum at the end of two hours are extremely small.<sup>1, 2</sup> Because the drug is so rapidly eliminated from the body, it has been found necessary to inject penicillin intravenously or intramuscularly at frequent intervals. The method of administration most widely used at the present time involves single intramuscular injections every three or four hours, day and night. The general type of blood curve attained by this



method is shown in Fig. 1. It will be noted that the amount of drug present in the blood serum is barely detectable during the third and fourth hours after each injection.<sup>3, 4</sup> Although intermittent intramuscular penicillin therapy is effective in the treatment of many severe bacterial infections,<sup>5-8</sup> the question may be raised as to whether intermittent peaks of penicillin concentration depress bacterial growth as effectively as does a continuous concentration of the drug.

The relative sensitivity of different bacteria to penicillin varies appreciably and may be measured *in vitro*.<sup>9</sup> It is doubtful whether favorable results can be expected in the treatment of clinical infections unless the amount of penicillin in the infected tissues is greater than the minimal concentration shown to exert an antibacterial effect in culture media.<sup>10</sup> If this premise be accepted, it would seem to follow that the most efficient method of administering penicillin is that which maintains continuously in the blood and tissues a concentration of drug greater than that needed to exert a bacteriostatic effect upon the offending organisms. It is logical to assume that the growth of the bacteria will escape from the action of the penicillin whenever the concentration of the drug falls below the minimal bacteriostatic level.

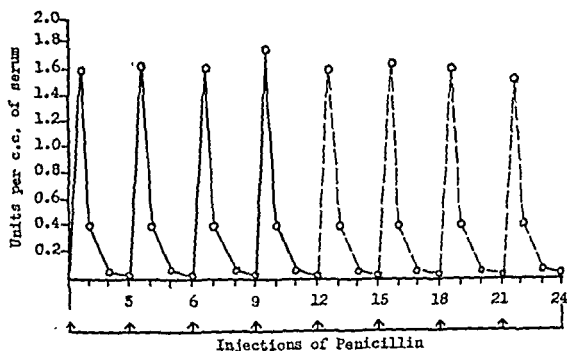


Fig. 1.—Blood level curve obtained by intermittent intramuscular injection of 20,000 units of penicillin given every three hours. Heavy lines, 0—0, represent actual observations.

Various methods have been advocated for maintaining more constant concentrations of penicillin in the blood. Some investigators have attempted to delay the absorption from the site of injection,<sup>11-13</sup> whereas others have administered substances which interfere with the excretion of the drug by the kidneys.<sup>14, 15</sup> The most promising method at the present time would appear to be that based upon the use of penicillin injected in a mixture of beeswax and peanut oil.<sup>12</sup>

With continuous drip therapy, absorption is slowed by the clamp on the tubing so that levels of penicillin in the blood show only small variations. Continuous intravenous drip<sup>16, 17</sup> has been employed but involves technical difficulties, particularly when the drug must be administered for more than a few days. Continuous subcutaneous drip has been found to be relatively unsatisfactory in that it yields significantly lower blood levels than does continuous intravenous administration.<sup>22</sup> Since preliminary reports suggest that continuous intramuscular therapy<sup>3, 18-21</sup> offers certain advantages over both of the methods just mentioned, it has seemed desirable to subject it to further clinical trial. The purpose of the present study has been twofold: first, to de-

termine quantitatively the blood levels attained by various dosages of penicillin administered by continuous intramuscular drip; and second, to evaluate the practicality of the method in the treatment of human infections.

#### METHODS

*Administration of Penicillin.*—The twenty-four-hour dose of penicillin\* was dissolved in 300 c.c. of normal salt solution and administered by a standard intravenous apparatus.† Instead of the usual glass needle adapters, short metal ones were used. Tunnel clamps‡§ were employed to adjust the flow of solution to 4 to 6 drops per minute. A No. 20 standard gauge 1½-inch needle was inserted deep into the vastus lateralis muscle after preparation of the thigh with iodine and alcohol. Sterile gauze dressings were placed about the needle which was then firmly taped in place. The needle was usually left in place for two or three days before being changed to the opposite side. In one instance the needle was left in place for five days without any apparent deleterious effect. Little change occurred in the speed of flow when the clamps were once set, and it was not found necessary to watch the drip at frequent intervals.

*Dosage of Penicillin.*—All but one of the patients studied were given, on succeeding days, twenty-four-hour doses of 100,000, 160,000, 240,000, 400,000, and 800,000 units of penicillin. These dosage schedules corresponded to intermittent injections of 12,000, 20,000, 30,000, 50,000, and 100,000 units given every three hours. One patient, acutely ill with subacute bacterial endocarditis, was given penicillin continuously at the rate of 100,000 units every hour for a period of twelve hours.

*Selection of Patients.*—All but the one patient with bacterial endocarditis had early syphilis and were selected because they were relatively young and were free from significant cardiac and renal disease.

*Estimation of Blood Levels.*—Each test was set up as indicated in Table I. Blood serum was obtained by aseptic technique, and twofold dilutions were prepared by adding M/50 phosphate buffer.|| A single dilution in the series was selected according to the estimated penicillin level in the serum, and varying amounts of this dilution, as indicated in Table I, were placed in test tubes measuring 1 by 10 cm. Diluent was added to each tube to bring the volume to 2 c.c.

A fresh culture of a standard strain of *Staphylococcus aureus* (209)|| was obtained by seeding 30 c.c. of single strength broth<sup>23</sup> from stock cultures and

TABLE I. METHOD OF TITRATING PENICILLIN CONCENTRATIONS IN UNKNOWN SAMPLE OF SERUM (VOLUMES EXPRESSED IN CUBIC CENTIMETERS)

TUBE	BUFFER	INOCULUM	UNKNOWN
1	0	2.0	2.0
2	0.4	2.0	1.6
3	0.6	2.0	1.4
4	0.8	2.0	1.2
5	1.0	2.0	1.0
6	1.2	2.0	0.8
7	1.4	2.0	0.6
8	1.6	2.0	0.4

\*The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigation recommended by the Committee on Chemotherapeutics and Other Agents of the National Research Council.

†Manufactured by The Upjohn Co., Kalamazoo, Mich.

‡Suggested by Dr. Chester S. Keefer.

§Manufactured by The Harvard Apparatus Co., Dover, Mass.

||A fresh pipette was used for the preparation of each dilution.

¶This strain was obtained through the kindness of Dr. J. V. Cooke.

incubating twelve hours, at 37° C. One cubic centimeter of this culture was added to 100 c.c. of double strength broth<sup>23</sup> and 2 c.c. of the mixture were added to each tube in the series. Aseptic precautions were observed throughout.

A single sample of commercial penicillin\* containing 295 Oxford units per milligram was used as a standard. Dilutions of this sample were sealed in ampules and stored at -70° C. Repeated tests with the standard strain of *Staphylococcus aureus* (209) revealed that an amount of 0.02 units constantly inhibited growth. In each group of tests a control series of tubes was included, using the standard.

All tubes were shaken well and incubated at 37° C. for eighteen hours. Readings were made by shaking the tubes, holding them up to a strong light, and determining by inspection the last tube showing diminished turbidity (that is, bacteriostasis). In practically all instances the end point was clear as evidenced by independent readings made by several observers.

The quantity of serum in the tubes was found to have no demonstrable effect upon the end point of the test.<sup>24</sup> When normal human serum was substituted for the phosphate buffer, the end point was unchanged, although the bacterial growth in the tube was somewhat more luxuriant.

Since the smallest amount of serum producing inhibition of growth was known to contain 0.02 units of penicillin, the concentration of drug per cubic centimeter of serum was easily calculated.

## RESULTS

*Practical Advantages of Methods.*—The majority of patients studied preferred the continuous intramuscular infusion to intermittent intramuscular injections given every three hours, day and night. Sleep was not interfered with, and once the needle had been taped in place, the patient was able to move about without undue discomfort. The rate of flow was not disturbed by movement of the leg, and the infusion apparatus needed only a minimal amount of attention from the nursing and resident staff. In most cases the patient was asked to watch the drip bulb occasionally and report any significant change to the nurse.

*Serum Concentrations of Penicillin.*—The blood levels of penicillin obtained in four patients given continuous intramuscular therapy of varying doses are shown in Fig. 2. Although some variation occurred, the blood levels attained were relatively constant throughout the twenty-four hours. The higher doses were associated with higher blood levels, and very little overlapping of the curves was observed. There was some individual variation in that all the levels of E. J. were somewhat lower than those observed in other patients. In Fig. 3 the number of units of penicillin administered in twenty-four hours is plotted against the average drug level attained in the blood. It will be seen that the levels are essentially proportional to the dosage, the relationship between the two variables being approximately linear. One patient was given penicillin continuously at the rate of 100,000 units every hour for a period of twelve hours—the equivalent of 2,400,000 units per twenty-four hours. The levels maintained in this patient's blood are shown in Fig. 4 and have been plotted separately because the magnitude of the values necessitates a different scale. Even at this extremely high dosage it was found that the linear relationship between dose and blood level still obtained.

*Complications.*—The reactions encountered in the sixteen patients are shown in Table II. Ten patients developed relatively severe local inflammatory re-

\*Lederle Laboratories, Inc., Pearl River, N. Y.

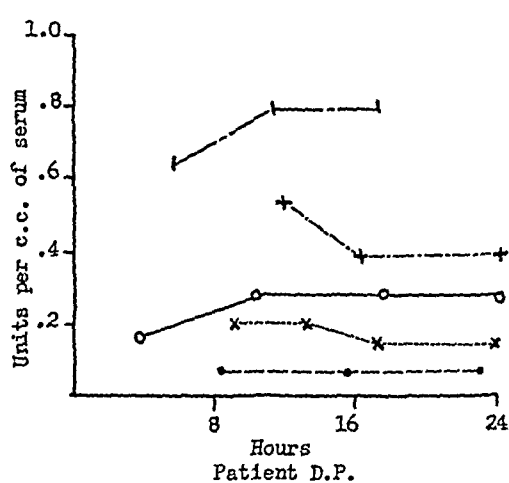
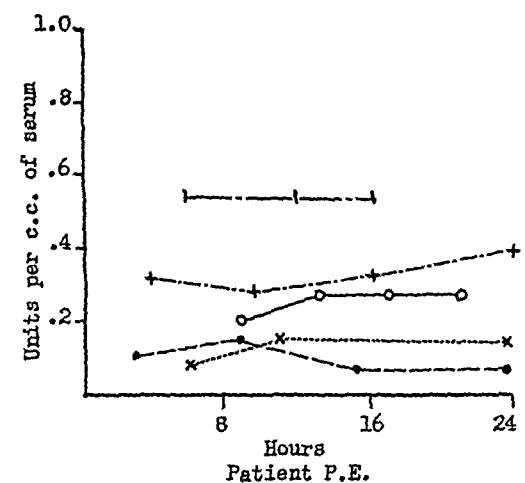
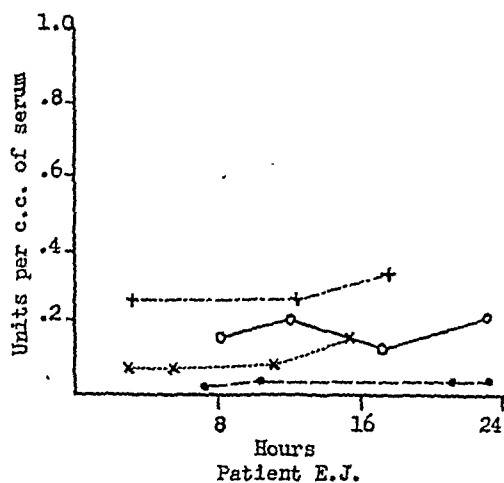
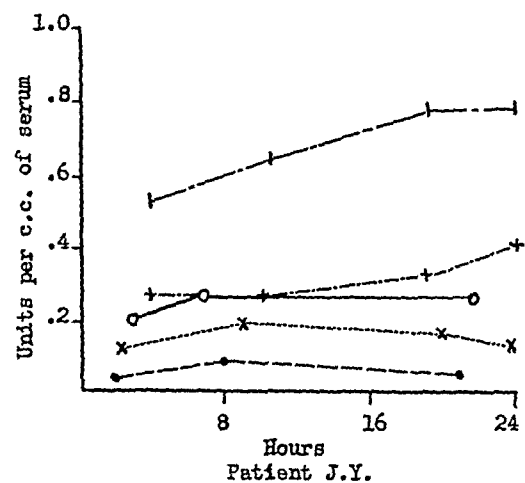


Fig. 2.

Fig. 2.—Concentrations of penicillin in the serum of patients receiving continuous intramuscular drip. Dosage in twenty-four hours: ●- - -●, 100,000 units; X...X, 160,000 units; 0-0-0, 240,000 units; +-.-.-+, 400,000 units; |—|, 800,000 units.

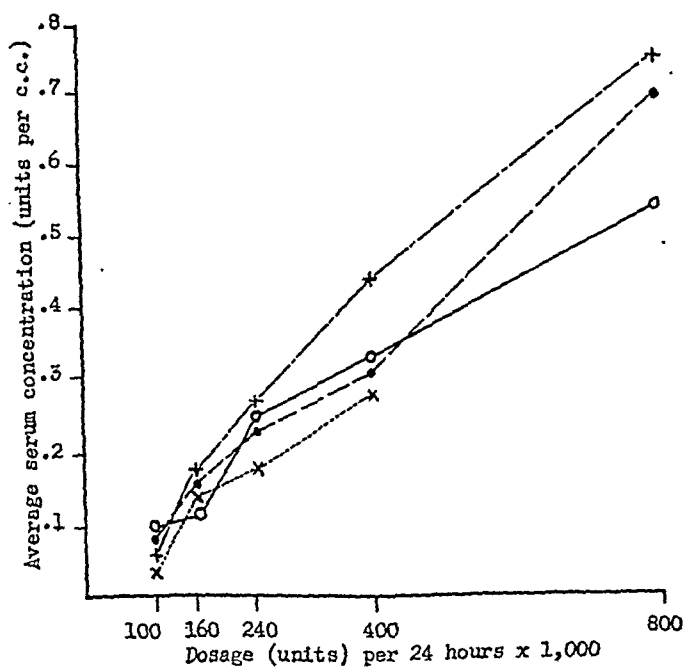


Fig. 3.—Relation of average serum level of penicillin to twenty-four-hour dosage. J. Y., ●- - -●; E. J., X...X; P. E., 0-0-0; D. P., +-.-.-+.

Fig. 3.

TABLE II. INCIDENCE OF REACTIONS TO CONTINUOUS INTRAMUSCULAR INJECTION OF PENICILLIN

Number of patients	16
Local inflammatory reaction	10
Fever	7
Leucocytosis	5
Phlebotrombosis	1
Subcutaneous emphysema	1
No complication	5

actions at the site of injection. In all but one case the reaction developed on the fifth to seventh day of treatment (Fig. 5) and consisted of severe local pain, redness, and local heat about the site of injection, usually involving the whole lateral aspect of the thigh. The opposite thigh never showed any swelling at the site of previous injection. The inflammatory process invariably subsided rapidly (usually within twenty-four hours) when the needle was removed. No evidence of bacterial invasion of the thigh was encountered, and no local therapy was required.

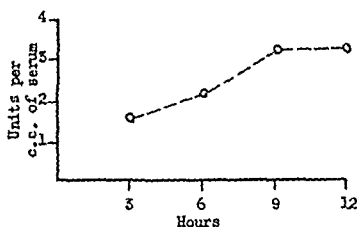


Fig. 4.—Serum levels of penicillin during continuous intramuscular administration of 2400,000 units in twenty-four hours

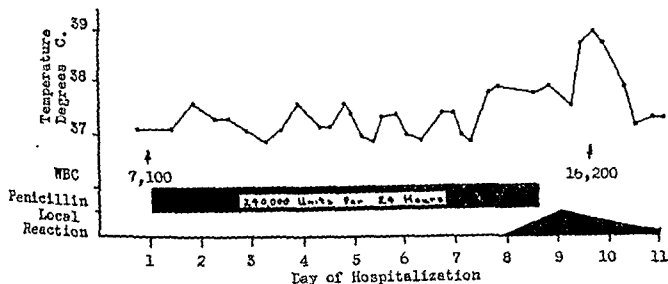


Fig. 5.—Occurrence of local inflammatory reaction, fever, and leucocytosis during continuous intramuscular administration of penicillin at the rate of 240,000 units per twenty-four hours.

The second most common reaction, which occurred in seven patients, was fever. The elevation in temperature was usually first noted on the sixth or seventh day (Fig. 5). When the continuous therapy was stopped, the fever subsided within twenty-four hours. All patients who developed fever showed evidence of a local inflammatory reaction, although the reverse was not true. The fever was frequently accompanied by leucocytosis of from 15,000 to 18,000 cells per cubic millimeter without significant shift in the differential count. In one case the fever and local reaction began on the second day, the fever ranging from 39 to 39.8° C.

A single patient developed phlebothrombosis of the femoral vein, probably as a result of inflammation of tissues above the vein. The needle in this case had been inserted into the anterior aspect of the thigh. One patient developed mild intramuscular and subcutaneous emphysema when the drip was allowed to run out, but this reaction subsided promptly.

#### CONCLUSIONS AND DISCUSSION

The results of the present investigation would appear to warrant two conclusions: first, that the continuous intramuscular administration of penicillin affords a practical method of maintaining relatively high concentrations of penicillin in the blood; and second, that with the penicillin preparations now available, the incidence of local reactions at the site of injection is too great to warrant routine use of the method at the present time. The blood levels attained by the continuous intramuscular drip are approximately the same as those reported with the continuous intravenous method.<sup>22</sup> This fact would seem to indicate that no significant amount of penicillin is destroyed at the intramuscular site of injection. It is of interest that the same linear relationship between dosage and blood level apparently obtains with both the intravenous and intramuscular routes. Blood levels as high as 2 or 3 units per cubic centimeter may be continuously maintained by the intramuscular method. It is suggested, therefore, that the method may be employed advantageously in the treatment of infections with relatively resistant bacteria.<sup>25</sup>

More than one-half of the patients treated in the present study developed local reactions at the site of the continuous intramuscular injections. Such local reactions have been described by previous observers.<sup>18-20</sup> It should be pointed out that relatively crude preparations of penicillin were used in the present study and in those previously reported. There is already suggestive evidence that the incidence of local reactions following intramuscular injections is inversely proportional to the purity of the penicillin employed.<sup>26</sup> It is conceivable that in the near future a crystalline form of penicillin will be available for therapeutic use. If more highly purified preparations of penicillin fail to cause local reactions at the site of injection, the continuous intramuscular method may be justifiably retained as a means of administering intensive penicillin therapy.

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## ACTINOMYCOTIC INFECTION OF THE SOFT TISSUES OF THE NECK: APPARENT CURE FOLLOWING LARGE DOSES OF PENICILLIN

### A CASE REPORT

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**T**HIS is a report of a case of actinomycosis of the soft tissues of the neck which was treated successfully with large doses of penicillin.

The patient, a white male officer, 28 years of age, was stationed in southern California. He was perfectly well until April, 1943, when he developed soreness in the region of the lower molars bilaterally. A diagnosis of impacted lower third molars was made and the teeth were extracted June 9, 1943, at his station hospital. Moderate swelling and tenderness were present on the left side following the operation but disappeared permanently after two weeks. The right side healed over a period of four weeks, but a sharply localized area of induration approximately 2 cm. in diameter remained in the soft tissues of the neck just inferior to the angle of the mandible in the posterior cervical triangle.

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## PROTHROMBIN IN PRESERVED BLOOD.

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**T**RANSFUSION of preserved blood has proved useful, especially where donors have not been available and it has been necessary to replace blood lost through hemorrhage.

Fresh blood transfusion is a usual procedure in instances where hemorrhage is due to disturbances of coagulation; in such cases blood transfusion is most important since the blood contains all the necessary factors for normal coagulation. Should preserved blood be administered in cases of hemorrhage due to hypoprothrombinemia, it is advisable to learn beforehand whether the blood to be transfused contains sufficient amounts of prothrombin, so that the patient's blood may be given, although momentarily, its capacity for normal coagulation.<sup>3, 8</sup>

Several investigators have studied the prothrombin stability of preserved blood for transfusions. Generally, after some days, prothrombin gradually diminishes in the blood and hence it cannot be used for transfusions designed to correct hypoprothrombinemia; however, there is considerable disparity in medical literature regarding the speed and degree of disappearance of prothrombin in preserved blood. Thus Belk and his co-workers<sup>1</sup> and Lord and Pastore,<sup>5</sup> by means of Quick's method,<sup>4</sup> did not find changes in the first five days, while Quick,<sup>6</sup> using his own method, reported a 50 per cent decrease in the first twenty-four hours. Ziegler and his co-workers,<sup>10</sup> using Smith's method,<sup>13</sup> found that the prothrombin progressively decreases during the first thirty-six days, while Rhoads and Panzer,<sup>7</sup> using a modified Quick's method, found that prothrombin diminishes to 25 per cent in seven days and to 20 per cent by the end of ten days. Warner and his collaborators,<sup>9</sup> employing Smith's procedure, found the following values for prothrombin: 91 per cent at the end of ten days and 50 per cent within twenty-one and twenty-five days. According to these authors, the difference in results is due both to the method of preserving blood and to the anticoagulant used. We feel that the methods employed to determine the prothrombin concentration are responsible for this disparity.

### EXPERIMENTATION

The blood used in this study was obtained from healthy adults in accordance with directions set by two of us<sup>11</sup>; that is, by mixing 9 volumes of blood with 1 volume of 0.1 N sodium oxalate (in this instance we employed sterilized sodium oxalate).

The blood thus collected was divided into two portions: one portion was aseptically stored in 2 c.c. ampules; the other was centrifuged and the separated plasma was kept in 1 c.c. ampules. These were kept, for the entire duration of the experiment, in the icebox at the Blood Transfusion Center.

Prothrombin determinations were carried out on those days indicated in Tables I and II. For fibrinogen solution, we used barium plasma prepared from the plasma of healthy human beings at the very moment the determinations were

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carried out. Thus, by diluting the plasma under study with fresh barium plasma, we were able to avoid the changes which could be attributed to the plasma itself.

In every instance, cultures were prepared from the plasma a few days after its separation; the cultures were invariably negative.

## DISCUSSION

During the first week we obtained hardly any significant changes in prothrombin time\* of the preserved blood samples (see Tables I and II and Figs. 1 and 3). This finding is in agreement with those of Belk and of Lord and Pastore. The prothrombin concentrations found with our method of determination are shown in Tables I and II and in Figs. 1 and 2; these, far from confirming a drop in the prothrombin concentration, show a remarkable increase which is maintained throughout the first week. From then onward a gradual increase is observed in prothrombin time and a drop in prothrombin concentration. It is interesting to point out that at the end of five months (Table I),

TABLE I. PROTHROMBIN CONCENTRATION IN A BLOOD SAMPLE PRESERVED DURING FIVE MONTHS AT 4° C

## SAMPLE I

DAYS	BLOOD		PLASMA	
	PROTHROMBIN TIME (SEC.)*	PROTHROMBIN†	PROTHROMBIN TIME (SEC.)	PROTHROMBIN (PER CENT)
--	19.0	101	19.0	101
1	19.0	124	19.5	143
2	17.5	210	18.0	--
5	18.5	98	21.5	80
9	26.5	95	22.5	104
13	26.0	104	25.5	105
19	27.5	105	26.0	109
22	28.5	73	25.5	--
33	33.0	36	37.0	47
37	32.5	34	41.5	42
54	35.0	28	40.5	36
155	No clotting in 2 min.	30	No clotting in 2 min.	37

\*0.2 ml. calcium-thromboplastin was added to a 0.1 ml. of plasma to determine the prothrombin time.

†Prothrombin concentration was determined according to the technique of Tanturi and Banti, with three plasma dilutions.

TABLE II. PROTHROMBIN CONCENTRATION OF BLOOD AND PLASMA PRESERVED AT 4° C.

## SAMPLES 1 AND 2

DAYS	SAMPLE 2				SAMPLE 3	
	BLOOD		PLASMA		PLASMA	
	PROTHROMBIN TIME (SEC.)	PROTHROMBIN (PER CENT)	PROTHROMBIN TIME (SEC.)	PROTHROMBIN (PER CENT)	PROTHROMBIN TIME (SEC.)	PROTHROMBIN (PER CENT)
--	18.5	113	18.5	113	19.5	102
1	19.0	146	19.0	130	19.0	128
2	18.0	180	19.5	190	20.0	175
3	20.0	238	17.5	180	19.5	206
4	21.5	146	21.0	126	21.0	143
6	23.0	133	20.5	103	27.0	127
7	24.0	107	23.0	102	23.0	111
8	27.0	109	30.0	93	28.0	99

Quick\* times 11 to 12.5 seconds) in normal plasma. Our error, since an experiment in the figures of the prothrombin time in normal blood is not similar to different manner. Quick determination (Quick's normal is from out with different dilutions of it may lead to considerable error gives rise to great varia-

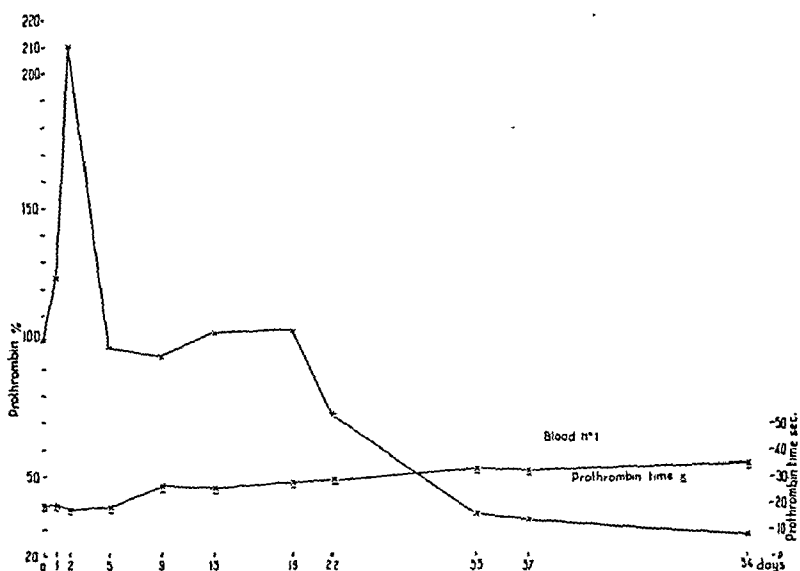


Fig. 1.—Prothrombin and prothrombin time variations in blood sample 1, stored for five months at 4° C.

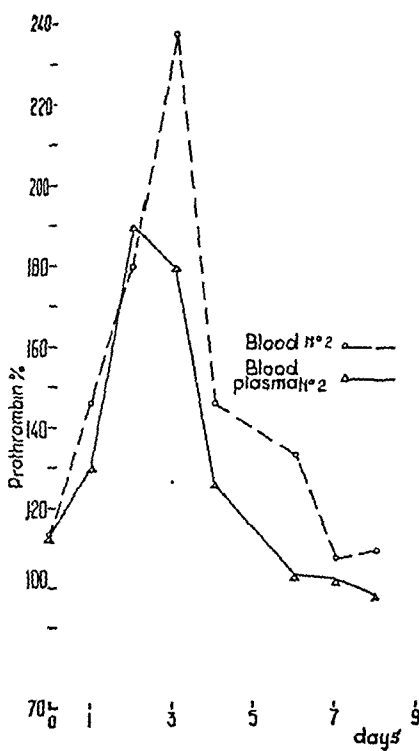


Fig. 2.—Prothrombin variations in blood sample 2, stored for eight days at 4° C.

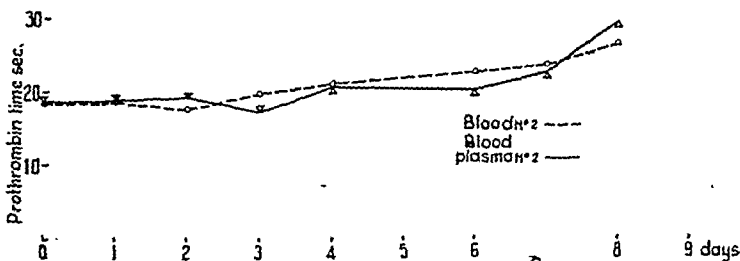


Fig. 3.—Prothrombin time variations in blood sample 2.



Tube 3 containing preserved plasma should have lost factor A and the addition of fresh plasma should have restored prothrombin to its normal constitution (AB complex). If Quick is right and the increase of prothrombin concentration is due to the addition of factor A, present in fresh diluting plasma and absent in stored plasma, we should never have been able to obtain the high prothrombin concentration found in our experiment; at the most, we should have had a concentration similar to that of fresh plasma.

We have already stated that prothrombin time does not vary in accordance with the prothrombin concentration (Tables I and II) and that this apparent contradiction during the first three days of storage could be attributed to an experimental error in the timing. We observe that after the fourth day prothrombin time increases gradually. This would mean a corresponding decrease of the prothrombin concentration. However, such is not the case. This discordance is possibly attributable to the formation of an antithrombin in stored plasma, as can be seen in the subsequent experiment utilizing plasma of the same subject.

TABLE IV. THROMBIN TIME IN FRESH AND STORED PLASMA

Fresh plasma	0.2 c.c. + Thrombin*	0.1 c.c. = 22 to 24 sec.
Stored plasma (5 days)	0.2 c.c. + Thrombin	0.1 c.c. = 43 to 44 sec.

\*Thrombin prepared after Eagle<sup>15</sup> in a 1/10 dilution.

We have also met the influence of this antithrombin when determining the thrombin time in plasma 2 of Table III. The fresh barium plasma showed a thrombin time of 22 seconds, while in stored barium plasma the time was 32 seconds. These results would appear to explain the differences found in the prothrombin concentration of fresh and stored diluting barium plasma.

The antithrombin action becomes less important when the stored plasma is diluted with fresh barium plasma; this explains the apparent disparity found between prothrombin time and concentration during storage.

The results of the foregoing experiments lead us to suggest that the increase of prothrombin concentration is not due to substances contained in the fresh diluting plasma.

So far, no investigator has pointed out the increase found during the first few days in the prothrombin concentration of stored blood and plasma. Perhaps this increase corresponds to a greater activity of the pre-existing prothrombin, which, under the conditions of our technique, becomes manifest as a greater concentration.

Crosbie and his co-workers,<sup>2</sup> using Howell's method through recalcification, found a shortening of the clotting time of blood during the first few days of storage. At the same time they were able to observe a rapid decrease in the number of platelets during the first few days. It is interesting to point out a coincidence in the shortening of the clotting time, a decrease of the number of platelets in the first few days of blood preservation (Crosbie), and an increase in prothrombin concentration found by us during the same period. It is possible that the reduction in the number of platelets results in the liberation of thromboplastin. The latter would render more sensitive the existing prothrombin, thus allowing a more rapid formation of thrombin at the moment of recalcification. This hypothesis should be the subject of further studies.

#### CONCLUSIONS

1. During the first three days, the prothrombin time of preserved plasma and blood undergoes slight variations. From then onward the time gradually

and continuously increases until the plasma, in spite of the addition of thromboplastin and calcium, becomes noncoagulable. This final noncoagulability is not due to an absence of prothrombin.

2. The prothrombin concentration, as determined by our technique, gradually increases and reaches its "maximum" at the end of three days, when it doubles its original value; later on it gradually decreases, and by the end of five months it reaches a 30 per cent concentration. A hypothesis is put forward that this increase in concentration registered in the first few days is due to prothrombin "sensitization."

3. After a certain period, transfusion of preserved blood or preserved plasma will not correct a prothrombin deficiency.

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# THE ISOLATION OF HEMOLYTIC STREPTOCOCCI FROM THROAT SWABS

## II. THE EFFICIENCY OF HUMAN AND RABBIT BLOOD IN STREAKED AND STREAKED-POURED PLATES

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WITH THE TECHNICAL ASSISTANCE OF HELEN LEONARD, A.B.

ALTHOUGH numerous observations have been made concerning the appearance of streptococcus colonies on various kinds of media, comparative data showing the relative value of different methods in the isolation of beta hemolytic streptococci from throat swabs are notably lacking. It has recently been shown<sup>1, 2</sup> that the detection of small numbers of hemolytic streptococci is facilitated by the preliminary incubation of throat swabs in an enrichment broth containing sodium azide and crystal violet. Two questions arose for which we were unable to find satisfactory answers in the literature. First, whether the number of hemolytic streptococcus isolations is materially affected by the kind of blood used, and second, whether plates which are inoculated in a manner to give deep colonies are more likely to be positive than those with surface inoculations.

The recognition of hemolytic streptococcus colonies depends largely upon observing the changes which they produce in the blood agar used for isolation. Following the demonstration of hemolysis by streptococci in rabbit blood agar by Marmorek<sup>3</sup> in 1902 and in human blood agar by Schottmüller<sup>4</sup> in 1903, it was shown that the erythrocytes of a number of different species were susceptible to hemolysis. Smith and Brown,<sup>5</sup> although not the first to use horse blood, based their description of alpha, beta, and gamma types on the appearance of colonies in horse blood agar. Consequently, horse blood has been considered as standard in many laboratories. Horse, human, or rabbit blood was recommended by Holman and co-workers,<sup>6</sup> who stated that the sharpest results were probably obtained with horse blood. Gilbert<sup>7</sup> reported that horse and human blood were equally satisfactory, but rabbit and sheep blood were found less suitable. Several strains from septic sore throat were hemolytic on sheep blood under anaerobic conditions only. Coffey<sup>8</sup> specifies either horse or human blood, while Schaub and Foley<sup>9</sup> state that rabbit, sheep, human or other blood may be used with little or no variation in the appearance of streptococcus colonies.

Becker<sup>10</sup> was one of the first to observe that strains of streptococci might be hemolytic on one kind of blood and not on others. Two strains which were apparently *Streptococcus pyogenes* were hemolytic on rabbit blood, but not on sheep or horse blood. A third strain hemolyzed horse and sheep cells but not goat, rabbit, or human cells. Smillie<sup>11</sup> called attention to the different types of hemolysis produced on the blood of different animals and stated that dog and guinea pig blood were not satisfactory. Brown<sup>12</sup> compared several strains of beta hemolytic streptococci in horse, human, and rabbit blood agar and found

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that variations in appearance on these bloods were quite insignificant. The appearance of alpha strains, however, was so dependent upon the kind of blood used that certain strains resembled the beta type on one kind of blood but not on another. Several strains of streptococci which hemolyzed human blood but not sheep blood and others which hemolyzed sheep but not human blood were recently reported by Traut and Johnson.<sup>13</sup> In none of these instances were the organisms identified serologically.

Sheep blood agar was used by Krumwiede and Kuttner<sup>14</sup> because of its inhibitory properties for the *Hemophilus* group. The elimination of *Hemophilus hemolyticus* colonies which often resemble those of hemolytic streptococci resulted in a medium which was partially selective for hemolytic streptococci. Human blood has a similar effect but is less inhibitory than sheep blood.<sup>9</sup>

It is, therefore, evident that some strains of streptococci do not produce the same appearance on plates prepared with various kinds of blood. To what extent these variations affect the efficiency of different bloods in isolating hemolytic streptococci is not clear. The kind of blood used in any laboratory depends partly upon its availability. Many laboratories do not have access to a source of horse blood. Rabbit blood is almost always obtainable, but when large quantities are required, the collection of the blood is somewhat time consuming. Human blood is, in most instances, available in the largest quantities, particularly in laboratories associated with hospitals. With the increasing prevalence of blood banks, many hospitals frequently have on hand sterile human blood which for various reasons cannot be used for transfusions.

The possible advantage of using poured plates in preference to the more convenient streaked plates for the isolation of hemolytic streptococci lacks convincing evidence. Krumwiede and Valentine<sup>15</sup> compared poured and streaked plates in culturing the throats of ten individuals and concluded that typical hemolysis may not be present on surface-streaked plates. The necessity of a standard method was recognized by Becker,<sup>16</sup> who suggested human blood-agar-streaked plates. Holman and associates<sup>6</sup> indicated the desirability of observing both surface and deep colonies but thought that surface inoculation was sufficient for routine purposes. Brown<sup>12</sup> showed that deep colonies are of more value than surface colonies in distinguishing beta, alpha, and alpha prime types. The value of poured plates in distinguishing serologic Groups A, B, and C was demonstrated by Rantz and Jewell,<sup>16</sup> who were able to correlate type of beta hemolysis with serologic group provided deep colonies were observed. Fry<sup>17</sup> observed several strains of Group A streptococci which formed nonhemolytic surface colonies when grown aerobically, but deep colonies in aerobic plates or surface colonies on anaerobic plates showed typical beta hemolysis.

Long and Bliss<sup>18</sup> recommended the use of poured rabbit blood plates for the isolation of minute beta hemolytic streptococci but presented no data to support the recommendation. Both surface and poured plates with horse blood were used by Rantz<sup>19</sup> in culturing a series of 345 excised tonsils and 298 throat swabs. Although complete data were not given, it was stated that 88 per cent of Group A strains from tonsils "were as readily isolated on surface as in poured plates." With throat swabs Rantz found that the number of isolations was influenced more by the skill of the technician than by the method of preparing the plates and concluded that skillfully prepared surface plates were nearly as satisfactory as poured plates. Dingle and co-workers<sup>20</sup> employed both streaked and poured plates in several hundred throat and sputum cultures but discontinued the poured plates because their use "did not increase significantly the growth of hemolytic streptococci of the beta type." No data were given.

The usual objection to the use of poured plates is that they are time consuming and inconvenient.<sup>19, 20</sup> Unless several dilutions of the material to be cultured are plated, there is always a risk of obtaining too many colonies for best differentiation. The necessity for pouring the plates individually at the time of inoculation is a distinct disadvantage. The first of these objections to poured plates is eliminated by the use of "double poured" plates as described by Perry and Petran.<sup>21</sup> In this method, thin agar plates are streaked in the usual manner and then a layer of blood agar is poured over the inoculated surface. Coffey<sup>8</sup> included these streaked-poured plates as an alternative to poured or streaked plates but stated that further data will be required to select the most practical method. Streaked-poured plates were tried by Rantz,<sup>19</sup> who found them to be satisfactory substitutes for poured plates.

It has been clearly demonstrated that the types of streptococci with reference to hemolysis can be more accurately recognized if deep rather than surface colonies are examined. It seems less certain, however, that plates prepared in a manner to produce deep colonies result in a greater number of hemolytic streptococcus isolations.

The data presented in this report provide a statistical comparison of plates prepared with rabbit and human blood and of streaked and streaked-poured plates with regard to their relative efficiency in demonstrating hemolytic streptococci on throat swabs from well children.

#### METHODS

Fresh beef heart infusion agar containing 1 per cent tryptose and adjusted to pH 7.6 before autoclaving was used throughout. For streaked plates blood was added in a concentration of 5 per cent. Rabbit blood was obtained by heart puncture and defibrinated by shaking with glass beads. It was stored in the refrigerator and usually was used within one week. Citrated human blood, discarded by neighboring blood banks because of positive serologic tests for syphilis, obtained in 500 c.c. amounts, was dispensed in 10 c.c. bottles and stored in the refrigerator for as long as six weeks. The amount of hemolysis which occurred in this time was not sufficient to alter the appearance of the blood agar or to interfere with the detection of hemolytic colonies.

In the preparation of streaked-poured plates, about 15 c.c. of beef heart infusion agar without blood were poured into each plate. These plates were stored in the refrigerator and were inoculated in the same manner as the streaked plates. Then 10 c.c. of melted agar containing 10 per cent blood were poured over the inoculated surface. In pouring this layer of blood agar, care was taken to disturb the inoculum as little as possible. Sometimes it was necessary to tilt the plate slightly in order to cover the bottom layer completely. A few surface colonies were desired and were always obtained, but too much mixing of the inoculum with the layer of blood agar made the colonies more difficult to fish. Best results were obtained when agar plates were prepared far enough in advance of inoculation so that the surface of the agar was dry. Experiments showed that using 5 per cent blood in both layers of agar made the plates too dense to read easily. Blood in the top layer only, in 5 per cent concentration, resulted in plates too light in color.

All plates were inoculated from overnight broth cultures of throat swabs prepared in the manner previously described.<sup>4</sup> Various ways of streaking plates were tried because the importance of obtaining an adequate sample of the broth culture without obscuring the results by overcrowded colonies was realized. In our hands the following method gave the desired result most consistently.



A straight needle was dipped into the well-mixed broth culture and streaked from fifteen to twenty times over about one-third of the plate. The plate was then turned 90 degrees and streaking continued at right angles to the first, again over one-third of the plate. The plate was turned again and the procedure repeated. Growth was rarely too heavy on the last section of the plate to be streaked.

Plates were examined after twenty-four to twenty-six hours' incubation at 35 to 36° C. Hemolytic colonies were fished to sections of rabbit blood agar plates. After incubation these subcultures were examined by inspection and Gram stain. Those resembling hemolytic streptococci were transferred to broth for confirmation by serologic grouping. Only one colony was fished from each plate unless the gross appearance suggested that more than one kind of hemolytic organism was present.

### RESULTS

*Comparison of Human and Rabbit Blood in Streaked Plates.*—Broth cultures of 199 throat swabs were streaked on both rabbit and human blood plates. The results are shown in Table I.

TABLE I. COMPARISON OF RABBIT AND HUMAN BLOOD PLATES STREAKED FROM BROTH CULTURES OF 199 THROAT SWABS

	BLOOD PLATES				DIFF. %	S.D. DIFF.	P
	RABBIT		HUMAN				
	NO.	%	NO.	%			
Hemolytic streptococci isolated	66	33.2	81	40.7	7.5	4.8	0.11
Group A	26	13.1	38	19.1	6.0	3.7	0.11
Hemolytic streptococci missed	16	8.0	1	0.5	7.5	2.0	0.0001
Group A streptococci missed	12	6.0	0	0	6.0	1.9	0.001
<i>H. hemolyticus</i> present	10	5.0	0	0	-	-	-
Suspected colonies present	85	42.7	118	59.3	16.6	5.0	0.001
Unconfirmed colonies present	21	10.6	37	18.6	8.0	3.6	0.016
Confirmed nonhemolytic colonies	8	4.0	2	1.0	-	-	-

Hemolytic streptococci of Groups A to G were isolated from eighty-one of the human blood plates and from sixty-six of the rabbit blood plates. Group A streptococci were obtained from twenty-six of the rabbit blood plates and from thirty-eight of the human blood plates. The difference in both cases is within the range of sampling error ( $P = 0.11$ ). The rabbit blood plates, however, failed to show hemolytic streptococci sixteen times when human blood plates from the same swabs were positive. Only once did the human blood plates fail when the rabbit blood plates were successful. Rabbit blood plates failed to isolate Group A streptococci twelve times or nearly one-third of all the A strains isolated. These comparisons of the two kinds of plates with respect to failures show differences which are statistically significant ( $P = 0.0001$  and  $0.001$ ).

The reason for rabbit blood plate failures is probably not that rabbit blood provides a less favorable medium than human blood. All strains grew readily on rabbit blood after isolation. The more likely explanation is that better hemolysis on human blood made the hemolytic streptococcus colonies easier to recognize. On eight of the rabbit blood plates, nonhemolytic colonies, subsequently identified serologically, were fished because of their morphology. Only two of the human blood plates showed such colonies. Both these strains belonged to Group B.

The greater amount of hemolysis on human blood is further reflected in the fact that suspected colonies which were not confirmed as hemolytic streptococci were fished from thirty-seven of the human blood plates. Unconfirmed

fishings were made from only twenty-one of the rabbit blood plates and these included ten plates which showed *H. hemolyticus*. Even when these ten plates are included, the difference between unconfirmed fishings from rabbit and human blood plates is unlikely to have arisen from chance ( $P = 0.016$ ).

Two factors which might have contributed to the difference in the results of using rabbit and human blood were (1) that the rabbit blood was defibrinated, while the human blood was prevented from clotting by the addition of sodium citrate, and (2) that the rabbit blood was always added to the medium within a few days after bleeding, whereas the human blood, being obtained in larger quantities, was stored for several weeks before being used. In order to control these factors, samples of human and rabbit blood were obtained, sodium citrate in a concentration of 1 per cent was added to a portion of each sample, and the remainder of each sample was defibrinated. Blood plates poured the same day the blood was obtained were streaked with pure cultures of several strains of hemolytic streptococci. After twenty-four hours' incubation, plates prepared with citrated blood were indistinguishable from those containing defibrinated blood of the same species. Hemolysis was definitely more marked on human than on rabbit blood agar.

Another difference in the appearance of Group A hemolytic streptococcus cultures on rabbit and human blood agar was repeatedly found in the morphology of the colonies. Cultures which formed flat, matt colonies on rabbit blood agar tended to be convex and glossy on human blood.

The broth containing a 1:15,000 concentration of sodium azide used for preliminary culture of the swabs did not completely inhibit *H. hemolyticus*. Since we have found *H. hemolyticus* in 41 per cent of swabs from a similar group of children cultured directly on rabbit blood agar,<sup>2</sup> the 5 per cent incidence here (Table I) represents a considerable reduction. Human blood<sup>3</sup> as well as the blood of certain animals<sup>14</sup> is somewhat inhibitory for organisms of the hemophilus group.

Rabbit blood plates failed most often when few hemolytic streptococci were present. The average number of hemolytic streptococcus colonies on the human blood plates from swabs which were negative on rabbit blood was eleven in comparison to an average of twenty-six on the same kind of plates from specimens which were positive on both rabbit and human blood.

*Comparison of Streaked and Streaked-Poured Plates.*—Streaked and streaked-poured plates were compared on 159 throat swabs (Table II). There was no significant difference between the two kinds of plates with respect to the total number of hemolytic streptococci isolated or the number which belonged to Group A. Six of the streaked plates were negative when the streaked-poured

TABLE II. COMPARISON OF STREAKED AND STREAKED-POURED HUMAN BLOOD PLATES FROM BROTH CULTURES OF 159 THROAT SWABS

	BLOOD PLATES				DIFF. %	S.D. DIFF.	P
	STREAKED		STREAKED- POURED				
	NO.	%	NO.	%			
Hemolytic streptococci isolated	62	39.0	66	41.5	2.5	5.5	>0.5
Group A	27	17.0	26	16.4	0.6	4.2	>0.5
Hemolytic streptococci missed	6	3.8	2	1.3	2.5	1.8	0.16
Group A streptococci missed	1	0.6	2	1.3	0.7	-	-
Suspected colonies present	78	49.1	83	52.2	3.1	5.6	>0.5
Unconfirmed colonies present	16	10.1	17	10.7	0.6	-	-
Average number of fishable colonies on positive plates	9	-	11	-	-	-	-

plates were positive, while only two of the latter missed hemolytic streptococci which the former detected, but this difference is probably not significant ( $P = 0.16$ ). Essentially the same number of each kind of plates showed suspected colonies which could not be confirmed after isolation.

The average number of isolated colonies thought to be fishable on the positive streaked plates was nine as compared with eleven in the streaked-poured plates. The latter figure was based on deep colonies only and does not take into account the surface colonies that were frequently present. Even when both deep and surface suspected colonies were present, the former were fished in order to determine the practicability of fishing deep colonies. In no case was there a failure to get growth in subculture when deep colonies were fished. Neither was there an excessive number of impure cultures.

As would be expected, the streaked-poured plates showed larger and clearer zones of hemolysis than the streaked plates. Although this difference might be an aid in reading the plates and in predicting the serologic group, it did not, in our experience, materially increase the number of streptococcus isolations.

TABLE III. COMPARISON OF RABBIT BLOOD STREAKED PLATES WITH HUMAN BLOOD STREAKED-POURED PLATES FROM BROTH CULTURES OF 159 THROAT SWABS

	BLOOD PLATES				DIFF. %	S. D. DIFF.	P
	RABBIT STREAKED NO.	%	HUMAN STREAKED- POURED NO.	%			
Hemolytic streptococci isolated	51	32.1	66	41.5	9.4	5.4	0.09
Group A	19	11.9	26	16.4	4.5	3.9	0.23
Hemolytic streptococci missed	14	8.8	0	0	8.8	2.3	0.0001
Group A streptococci missed	7	4.4	0	0	4.4	-	-
Suspected colonies present	64	40.3	83	52.2	11.9	5.6	0.04
Unconfirmed colonies present	13	8.2	17	10.7	2.5	3.2	0.42
Positive for <i>H. hemolyticus</i>	8	5.0	0	0	5.0	-	-

Data on rabbit blood streaked plates are also available for comparison with the human blood streaked-poured plates (Table III). The difference in the number of plates showing hemolytic streptococci by these two methods is greater than the difference shown in Table I. The advantage of the streaked-poured plates with human blood over rabbit blood surface plates with respect to total and Group A streptococci isolated, however, is not statistically certain. On the other hand, no swabs were found positive by the latter which were missed by the former, while rabbit blood streaked plates failed fourteen times when the streaked-poured plates were positive. This difference appears to be highly significant ( $P = 0.0001$ ). There was a difference of only 2.5 per cent in the two kinds of plates which showed hemolytic colonies not confirmed. Excluding the eight rabbit blood plates which showed *H. hemolyticus*, this difference becomes 7.5 per cent, which is probably significant ( $P = 0.007$ ). The increased hemolysis due to human blood as well as that due to the presence of deep colonies probably contributed to this difference.

#### DISCUSSION

Although our data indicate that human blood plates are slightly superior to rabbit blood plates as far as demonstrating the presence of hemolytic streptococci is concerned, there are three points to be considered in connection with the use of human blood. (1) Cultures on human blood agar showed more suspected colonies which were not confirmed as hemolytic streptococci than did the rabbit

fishings were made from only twenty-one of the rabbit blood plates and these included ten plates which showed *H. hemolyticus*. Even when these ten plates are included, the difference between unconfirmed fishings from rabbit and human blood plates is unlikely to have arisen from chance ( $P = 0.016$ ).

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## PROGRESS

### CLINICAL SIGNIFICANCE OF SERUM PROTEIN (ALBUMIN AND GLOBULIN) CHANGES

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AS THE result of much work during recent years it has become clear that a knowledge of the serum protein level is of considerable significance in the diagnosis and treatment of many medical and surgical conditions. Thus, important changes of the serum protein concentration may be encountered in Bright's disease, liver disease, and malnutrition, in conditions associated with plasma depletion, including burns, hemorrhage, tissue trauma, peritonitis, and intestinal obstruction, and in others. It is little wonder, then, that the clinical laboratories are frequently called upon to do serum protein determinations.

Before discussing certain specific examples of altered serum albumin and globulin concentrations encountered clinically, a brief consideration will be given to the subject of plasma protein formation and to the physiologic role of plasma protein in controlling fluid exchange between the plasma and the interstitial fluid compartment.

#### METABOLISM OF PLASMA AND TISSUE PROTEINS

*Normal Range of Variation of Serum Proteins.*—Although, for practical purposes, only the two protein components albumin and globulin are usually determined clinically, evidence points to the presence in serum of a number of different globulins and probably more than one albumin. As is well known, globulins can be separated readily from albumins by precipitation at salt concentrations above a certain point. For example, in the familiar Howe procedure<sup>1</sup> the globulins are precipitated by a 22 per cent sodium sulfate solution. Further, when the sodium sulfate is present at concentrations of 13.5, 17.4, and 21.5 per cent, respectively, three globulin precipitations are realized and these have customarily been termed euglobulin, pseudoglobulin I, and pseudoglobulin II. The significance of the latter globulin fractionation has been obscured somewhat by recent electrophoretic studies of serum,<sup>2-5</sup> which showed that aside from albumin, serum normally contains three globulin components; namely,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulin. Although relatively few data are available at present concerning the changes of these globulin components in health and disease, there would appear to be significant disturbances in their absolute and relative amounts in certain diseases exhibiting hyperproteinemia.<sup>3, 6</sup>

From the findings of several groups of workers<sup>7, 8</sup> it would appear that in normal adults the total serum protein concentration may vary between 6.0 and 8.0 Gm. per 100 c.c.; albumin, between 3.6 and 5.5 Gm. per 100 c.c.; and globulin, between 1.4 and 3.5 Gm. per 100 c.c. The great majority of the figures for the total serum protein concentration apparently fall within the narrower limits of 6.1 and 7.7 Gm. per 100 c.c.<sup>4, 8, 9</sup> The ranges of variation of serum albumin and globulin depend somewhat upon the method of estimation. Thus, Robinson, Price, and Hogden<sup>10</sup> found, in studying the determination of the

albumin and globulin fractions of blood serum by the Howe method,<sup>1</sup> that a variable amount of albumin is adsorbed by the filter paper. It was found, further, that within rather wide limits the amount of albumin adsorbed is independent of the concentration of albumin but is dependent upon the type and quantity of filter paper. Such adsorption of albumin causes an error in the determination of the albumin concentration. Indirectly, therefore, it also causes an error in the globulin figure, since the latter is taken as the difference between the total serum protein concentration and the albumin concentration. By employing the Howe method and introducing the precautions necessary to avoid loss of filtrate protein through adsorption on filter paper, Gutman and co-workers<sup>6</sup> found the albumin to vary between 4.7 and 5.7 Gm. per 100 c.c. and the globulin to vary between 1.3 and 2.5 Gm. per 100 c.c. in thirty-six normal subjects. In other words, the range of values for albumin was found to be somewhat higher and that for globulin to be somewhat lower than those usually cited.

It seems well established<sup>6</sup> that significantly higher values for total serum protein and albumin may be encountered in healthy persons, for example, in laboratory workers and students, rather than in hospitalized control subjects. There is evidence<sup>8</sup> that at birth the total serum protein concentration and protein fractions, especially the globulin, are lower than those observed in adults. However, the various values rise shortly after birth so that from 3 to 6 months of age<sup>8, 11</sup> they fall within the normal ranges of variation for adults.

*Plasma Protein Formation.*—It would appear that the normal organism has a considerable reserve capacity for forming new plasma proteins since in the absence of infection or disease and in the presence of an adequate protein intake, an acute depletion of plasma protein is rather quickly corrected. Conclusive information is still lacking regarding the origin of plasma proteins, especially albumin and globulin. However, Whipple and his associates<sup>12-15</sup> and others<sup>16</sup> have presented evidence that the liver is the site of plasma protein production or, at least, is intimately concerned with plasma protein fabrication. The belief that the albumins come from liver seems quite well established. However, there still remains some doubt in the case of the globulins since, even although the liver may play an important role in contributing them, there is evidence<sup>17, 18</sup> that the reticulo-endothelium and other cells may also be sites of origin of globulins.

The ability of the normal organism to fabricate new plasma protein has been studied experimentally by determining the plasma protein regeneration following protein depletion induced either by plasmapheresis or by the administration of a diet very low in protein. The former method of plasma protein depletion has been employed extensively by Whipple and his co-workers.<sup>14, 20</sup> In this method the plasma protein of a dog on a basal diet is reduced and maintained by repeated plasmapheresis at a level close to 4 Gm. per 100 c.c. This subnormal level of the plasma protein is assumed to act as a constant and maximal stimulus for the production of new protein. The plasma protein production is then measured by determining the amount of protein which must be removed by plasmapheresis to maintain the plasma protein concentration at the reduced level. In such experiments it was found that when the plasma protein depletion was begun, the weekly output of protein was high at first and then gradually fell to a basal output. However, when the basal diet and other factors were kept uniform, the basal output of protein was found to remain relatively constant and such a standardized animal could then be employed to test the effect of diet and of other factors on protein regeneration.

concentration at the time of first observation may be misleading and a false security may be placed in a normal concentration. If there has been a simultaneous loss of plasma volume, the loss of total circulating plasma protein may be obscured. Consequently, with a correction of dehydration by saline, intravenously, the serum protein concentration may fall below the "critical level" and edema may result. It is now generally appreciated that in such patients fluid replacement entails the use of plasma (or plasma substitutes) as well as saline.

#### SERUM ALBUMIN AND GLOBULIN CHANGES ENCOUNTERED CLINICALLY

*Renal Disease.*—That the serum protein concentration may be found altered in various types of Bright's disease is well established. In the non-hemorrhagic degenerative Bright's disease or nephrosis the serum protein concentration may be decreased markedly and the deficit is due almost entirely to albumin.<sup>56, 58-60</sup> In extreme cases the plasma albumin concentration may fall below 1 Gm. per 100 c.c., to be accompanied by marked edema. The tendency to edema formation in nephrosis is closely related to the serum protein (albumin) concentration and hence its determination is very important clinically. Recent attempts<sup>41, 61, 62</sup> to correct the hypoalbuminemia of nephrosis by injections of concentrated human blood serum or concentrated human serum albumin have yielded results which are not too encouraging, but further careful investigations seem warranted.

Hemorrhagic nephritis or glomerulonephritis may be accompanied by serum protein deficits which approach those observed in severe nephrosis. In acute glomerulonephritis the serum protein concentration may remain within normal limits throughout the illness if the course is mild.<sup>60, 63</sup> Such cases may exhibit rather marked edema despite the fact that the serum protein concentration is within the normal range of variation. This represents an outstanding example of the lack of relationship between the serum protein level and tendency to edema, and it has been generally held that in acute glomerulonephritis the capillaries are probably injured and abnormally permeable to protein, thereby increasing the tendency to edema. However, recent observations by Warren and Stead<sup>64</sup> have left some doubt that this is true. The latter authors examined the protein content of edema fluid collected from the subcutaneous tissues of the leg and from the sacral region of patients with acute glomerulonephritis. It was found that the values for the total protein content, which varied between 0.1 and 1.0 Gm. per 100 c.c., did not differ significantly from those obtained on the edema fluid of patients with congestive heart failure. The authors expressed the view that, with the evidence at hand, the cause of edema in acute nephritis is water and salt retention secondary to a disturbance in renal function and not the result of diffuse capillary damage throughout the body. In any event, unless a serum protein deficit ensues, the edema is usually only of short duration. In the more severe cases and as the condition is prolonged a serum protein deficit occurs wherein the albumin concentration may fall to a level definitely below 2 Gm. per 100 c.c.<sup>58, 60, 63</sup> In these patients edema is invariably present when the albumin concentration is below 2.2 Gm. per 100 c.c.,<sup>63</sup> and the tendency toward edema remains until the serum protein rises above the critical level.

Chronic glomerulonephritis, the nephrotic type, or chronic active hemorrhagic nephritis is accompanied by a decrease in the serum protein concentration, representing chiefly a deficit of albumin.<sup>59, 60</sup> The presence or absence of edema in this condition appears directly dependent upon the serum protein



level. As the condition progresses from the acute or the chronic stages to terminal glomerulonephritis, there is a tendency for the serum protein concentration to increase to normal. This apparently happens in about 50 per cent of the cases,<sup>60</sup> although lowered serum protein concentrations are not infrequently encountered.<sup>62</sup>

*Liver Disease.*—The alterations of the serum proteins in hepatic disease have attracted the attention of many workers and the change most frequently encountered (particularly in cirrhosis of the liver) is a deficit of the albumin fraction.<sup>8, 60, 71</sup> The globulin concentration is generally elevated so that in many patients the total serum protein concentration is within the normal range of variation. Not infrequently, however, the deficits of albumin are greater than the increases of globulin so that the total serum protein concentration is lowered. Although malnutrition may be a factor contributing to the hypoalbuminemia encountered,<sup>8</sup> various workers<sup>67, 70, 71</sup> have emphasized the feature of an impairment in the fabrication of albumin.

*Malnutrition.*—The importance of malnutrition (inadequate protein intake and/or improper assimilation) as a predisposing factor toward the hypoalbuminemia observed in certain diseased conditions has become generally recognized.<sup>8, 72-74</sup> The hypoproteinemia of malnutrition is due to a deficit of the albumin fraction; the globulin (except in cases complicated by infection) remains relatively constant. As was pointed out, experiments on dogs have revealed the fact that tissue protein as well as serum protein becomes depleted when the protein intake is inadequate. With a fall of the serum albumin, the plasma volume also becomes reduced, and this frequently obscures the magnitude of total circulating plasma albumin loss.

*Plasma Protein Deficiency in Trauma.*—A fundamental problem in trauma (including hemorrhage, burns, "crush-injuries," peritonitis, and intestinal obstruction) is the correction or prevention of plasma (protein) depletion.

*Hemorrhage:* It is now clear that an understanding of the pathologic physiology resulting from acute blood loss is of great importance in the management of many medical and surgical patients. In the past, attention of the clinician has been directed principally to the restoration of blood cells and hemoglobin following acute hemorrhage. However, recent studies indicate that restoration of plasma volume and total circulating plasma proteins are equally important phenomena since these processes help to re-establish the hemodynamics of the peripheral circulation so that the hemoglobin remaining in the circulation can carry out its function of transporting oxygen to the tissues.

From studies which have been carried out on normal dogs<sup>75-82</sup> and on human subjects<sup>83, 84</sup> it would appear that following a single non-fatal hemorrhage there is a prompt, but gradual, inflow of fluid and protein into the circulation. The initial increase of plasma volume is apparently the result of the addition of fluid relatively low in protein, and in from several hours to some seventy hours the plasma volume may exceed the control level. In general, it has been found that the plasma protein (albumin) is restored more slowly than the plasma volume and may still be subnormal at the end of several days. Apparently the slow return of plasma volume cannot be attributed to a lack of fluid in the body with which to dilute the blood, since physiologic saline, when given intravenously, does not maintain blood dilution but leaves the circulation rapidly.<sup>77, 84</sup> Likewise, the even slower return of the plasma albumin has been taken as evidence against the existence of a reserve of preformed plasma protein which

can enter the blood stream in the first few minutes after hemorrhage.<sup>80</sup> It seems more likely that the protein entering the circulation under these circumstances represents protein stored as a part of tissue protein outside the circulation or that protein building materials are readily available for the fabrication of new protein. Since considerable evidence points to the view that there is a continuous traffic of plasma protein leaving and entering the circulation and that there is a continuous exchange between the plasma proteins and the tissue protein stores, the initial increase of total circulating plasma albumin following hemorrhage may be a redistribution of mobile plasma protein. It is of some interest in this connection that dogs made hypoproteinemic by diet are capable, without intravenous fluids, of restoring the plasma volume to the prehemorrhage level and of adding a small amount of plasma albumin to the circulation following a single nonfatal hemorrhage.<sup>85</sup>

From experiments in which shock has been produced by graded hemorrhage<sup>80, 86</sup> there appears to be a deficiency in the ability of the organism to dilute the blood and to add plasma protein to the circulation. In fact, as evidenced by a rise of the hematocrit, there may be an actual loss of plasma fluid and plasma protein from the circulation when shock develops.<sup>86</sup> On the other hand, there is evidence<sup>87-89</sup> against the existence of an increased permeability of the general capillary bed to plasma protein in hemorrhagic shock. The progressive decline of the plasma volume in hemorrhagic shock might well be attributed to a progressive fall in the volume of actively circulating plasma associated with peripheral stagnation.<sup>87-89</sup>

The intravenous injection of physiologic solution of saline may be effective in restoring the blood pressure following mild hemorrhage. It is quite ineffective, however, in patients with surgical shock and profuse hemorrhage. Even following nonfatal hemorrhage the injected physiologic solution of saline rapidly leaves the circulation<sup>77</sup> and in shock it may actually aggravate the escape of plasma protein from the circulation.<sup>80</sup>

**Burns:** Following severe burns there is usually a marked negative nitrogen balance<sup>90-92</sup> which may persist for days. In addition to an increased urinary nitrogen output, the nitrogen loss in the exudate from surface burns may represent a sizable per cent of the total nitrogen lost from the body.<sup>92</sup> An outstanding feature of burn shock is the extreme degree of hemoconcentration associated with a diminished plasma volume, attributable to a leakage of plasma from the capillaries in the burned area and in the tissue surrounding it. Increased capillary permeability appears to exist only in the regions near the burn and apparently does not include the capillaries in an area remote from the burn.<sup>88, 89, 93, 94</sup> The serum protein concentration may show little change but is generally reduced. On the other hand, the total amount of circulating plasma albumin is always reduced in severe burns.

**Crush Injuries:** The release of compression, due to fallen beams, masonry, or debris pressing heavily upon some part of the body, usually an extremity, has been found to be followed in a few days by oliguria which in certain cases progresses to anuria and death.<sup>95, 96</sup> Neither the duration of the compression nor the amount of the body involved appears to be directly proportional to the ultimate outcome.

It has become evident that the injury results in degeneration and necrosis of the muscle. Apparently as the result of tissue trauma there is an altered permeability of the capillaries and plasmalike fluid escapes into the injured area. The loss of plasma volume leads to hemoconcentration and fall of blood

pressure. The serum proteins may be increased at first and then return to normal. In some cases the serum proteins become reduced, particularly following the administration of crystalloid solutions, and in the latter instance there may be widespread edema.

Although the circulatory effects of crush injuries are understandable, the more important sequela of this type of trauma, renal failure ("crushed syndrome"), is more difficult to interpret. When oliguria occurs, the urine is brownish in color and complete suppression of urine ultimately supervenes. Apparently various products from the necrotic muscle [myohemoglobin (myohematin), potassium, phosphate, and possibly other substances] escape into the circulation when it is re-established locally following the release of compression. The release of such products may contribute to the severe renal impairment with death in uremia which follows in about 66 per cent of the patients.<sup>97-99</sup>

*Peritonitis and Intestinal Obstruction:* Clinical and experimental studies<sup>97-99</sup> have demonstrated that in spreading peritonitis and intestinal obstruction hypoproteinemia affecting primarily the albumin fraction is of frequent occurrence. In both of these conditions there is a loss of fluid containing protein in the walls of the intestines and the peritoneal cavity. There is, therefore, a decrease of plasma volume which may be detected by an increase of the hematocrit. In many ways the pathogenesis is similar to that presented by severe burns.

*Hyperproteinemia:* A number of years ago Wu<sup>100</sup> noted that in kala-azar the total serum protein concentration may be increased to 9 to 10 Gm per 100 c.c. The increase was found to be due to a rise of the globulin fraction, the albumin, on the other hand, being definitely reduced. With subsequent accumulation of data it seems permissible to draw the generalization that whereas hypoproteinemia is due chiefly to a deficiency of albumin, hyperproteinemia is due to an increase of the globulin fraction. Kagan,<sup>17, 18</sup> reporting observations carried out on a large series of cases, with special attention to the incidence of hyperproteinemia, concluded that hyperglobulinemia appears principally in diseases involving the bone marrow, chronic infections, diseases involving the liver, and in dehydration. A number of authors have reported the serum globulin to be increased significantly in patients with multiple myeloma,<sup>6, 18, 101-103</sup> chronic infections of various origins,<sup>6, 8, 18, 75</sup> lymphogranuloma inguinale,<sup>103, 104</sup> Boeck's sarcoid,<sup>103, 105</sup> and cirrhosis of the liver.<sup>6, 104</sup> In acute dehydration the serum protein concentration becomes increased, both the albumin and globulin fractions being affected. Not infrequently, however, the increased serum protein level in dehydration is due to increased globulin, the albumin concentration being within the normal range of variation or even reduced.<sup>18</sup>

Although further work appears desirable, recent electrophoretic studies of sera from patients exhibiting hyperproteinemia are of interest. Attempts to correlate the serum electrophoresis findings with protein concentrations as determined by the salting-out technique of Howe<sup>1</sup> have raised doubt that the fractionation of serum based upon solubility in salt solutions represents a clear-cut division. Thus, in normal human serum<sup>6</sup> the electrophoretic pattern was found to be essentially unchanged after precipitation in 13.5 per cent sodium sulfate (after the removal of euglobulin). About one-half of the gamma, one-fourth of the beta, and none of the alpha components were found to be removed by precipitation in 17.4 per cent sodium sulfate (after euglobulin and pseudoglobulin I had been removed. All of the gamma, about three-fourths of the beta, and one-fourth of the alpha components were found to be removed by precipitation in 21.5 per cent sodium sulfate (after everything except the albumin had

supposedly been removed). In other words, a considerable amount of the  $\alpha$ - and  $\beta$ -globulins were found to have remained with the albumin. Longsworth, Shedlovsky, and MacInnes<sup>3</sup> observed the electrophoretic patterns of sera from patients showing a variety of pathologic conditions, with fever in common, and all showed an increased  $\alpha$ -globulin content. In patients with portal cirrhosis the  $\beta$ - and  $\gamma$ -globulins were found to be increased<sup>5</sup> and the  $\gamma$ -globulin strikingly so when ascites was present. Gutman and co-workers<sup>6</sup> carried out serum protein studies by the Howe method in thirty-eight patients with multiple myeloma and electrophoretic analyses in ten patients. As the result of these studies the authors concluded that most myelomatous sera fall into one of the following three classifications: (1) sera with hyperglobulinemia due to an increase chiefly in the Howe euglobulin fraction, partly to an increase of the pseudoglobulin I fraction and, exhibiting electrophoretically an increase in the gamma components; (2) sera with or without hyperglobulinemia giving a variety of anomalous patterns by the Howe or electrophoretic methods, for the most part due to Bence-Jones proteinemia; and, (3) sera of apparently normal composition with respect to serum proteins.

#### SUMMARY

From evidence which is now available it would appear that the normal organism has a marked capacity to synthesize plasma proteins under conditions of stress. The "building blocks" for plasma protein fabrication may be supplied as free amino acids, hydrolyzed protein, or plasma either directly by vein or orally. For maintenance of nitrogen balance, however, it is necessary to supply in optimal amounts all of the essential amino acids. Infection and intoxication appear to retard plasma protein regeneration.

There is much to support the view that the plasma proteins are in a continuous traffic, leaving and entering the circulation and undergoing a continuous exchange with the tissue proteins. During periods of restricted or inadequate protein intake, the excess nitrogen lost from the body is contributed both by the plasma proteins and tissue proteins; the ratio of the loss of plasma albumin to tissue protein is approximately 1 to 25 or 30. With the resumption of an adequate protein intake, plasma albumin and tissue protein are restored in approximately the same ratio. This emphasizes the practical point that in patients with serious protein depletion, primarily due to malnutrition, the correction to normal may require the administration of large amounts of protein and a considerable period of time may be necessary.

Under normal conditions the escape of plasma protein (albumin) from the circulation is sufficiently restricted so that it can carry out its important function of sustaining the plasma volume. In "shocklike" conditions (including burns, crushing injuries, peritonitis, and possibly severe hemorrhage) the integrity of the membrane becomes disturbed. This results in a loss of protein and fluid from the plasma, which may lead to serious consequences.

Significant disturbances of the serum protein concentration may be frequently encountered clinically. It seems permissible to draw the generalization that whereas hypoproteinemia can be attributed chiefly to a deficiency of albumin, hyperproteinemia is due to an increase of the globulin fraction. Hypoproteinemia may be encountered, for example, in Bright's disease, malnutrition associated with various conditions, liver disease, and various conditions associated with plasma protein loss. It would appear that hyperproteinemia is not

so rare as was once believed. Hyperproteinemia (hyperglobulinemia) may be encountered principally in diseases involving the bone marrow, chronic infections, diseases involving the liver, and in dehydration.

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## LABORATORY METHODS

### MODIFICATION OF THE HAMILTON OPTICAL MANOMETER

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THE metallic membrane manometer, originally designed by Hamilton, Brewer, and Brotman,<sup>1</sup> was, in several respects, unsatisfactory for the work to be done in the Research Section of the School of Aviation Medicine.

1. Adjustment by eight set screws was time consuming and the suspension from a horizontal bar rendered the instrument vulnerable to extraneous vibrations.

2. Sensitivity was so limited that the long optical arm made operation of the instrument in a limited space complicated and awkward.

3. The assembly was composed of three units: a source of light, a camera, and a bank of manometers, each individually mounted and somewhat difficult to align.

4. The source of light consisted of a 500-watt, monophane, filament projection lamp enclosed in a ten-inch, cubical brass box with an adjustable slit for casting the beam of light on the mirrors. This arrangement was found to be unsatisfactory because of the excessive heat generated and because of the leakage of light from vents in the box. Incorporation of a fan and motor blower was unsatisfactory because of the vibration created.

5. Finally, cameras for 12 cm. bromide paper are difficult to purchase and quite expensive. The one constructed in this laboratory is simple, easily assembled, and entirely satisfactory.

The electrical capacitance diaphragm manometer described by Lilly<sup>2</sup> was considered for use, but the need for multiple simultaneous records and therefore multiple pieces of this apparatus nullified one important requirement of the instrument for the research to be done; namely, compactness.

#### DESCRIPTION OF APPARATUS

*The Assembly.*—A table for the manometers measuring 30 by 30 inches was so constructed that it could be immobilized on tripod supports or moved about on casters. To prevent parallax and to facilitate focusing, the height of the table is adjustable in order to bring the mirrors on a level with the slit of the camera. Although the size of the table may appear to be excessive, it was found that the space on it in front of the manometers is convenient for holding antiseptics, sponges, and other necessary items for an experiment. The base of each manometer carrier was attached to a tripod pan and tilt head (Fig. 1). These in turn were secured to a cast iron bar, 1 $\frac{1}{4}$  inches high, at one side of the table top. A manometer may be quickly aimed at the camera and fixed in position by simple manipulation of the adjustment handle of the pan and tilt

<sup>1</sup>From the Department of Medicine, Army Air Forces School of Aviation Medicine, Randolph Field, Texas.

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head. The housing, clamped within the carrier, is a circular brass tube with eight set screws supporting the manometer.

*Frequency and Sensitivity of Apparatus.*—Sensitivity may be augmented in three ways: by increasing the distance of the optical arm (distance from light source and camera to the manometer), by decreasing the thickness or type of membrane, and by increasing the diameter of the manometric system and hence the cross-sectional area through which a force may act upon the membrane. It is impractical to lengthen the optical arm, particularly if the instrument is to be employed where conservation of space is an important item. Membranes with a thickness of 0.001 inch or less tend to buckle when exposed to arterial pressures. Enlarging the diameter of the system increases the effective mass and decreases frequency. Hamilton, therefore, set the optimal optical

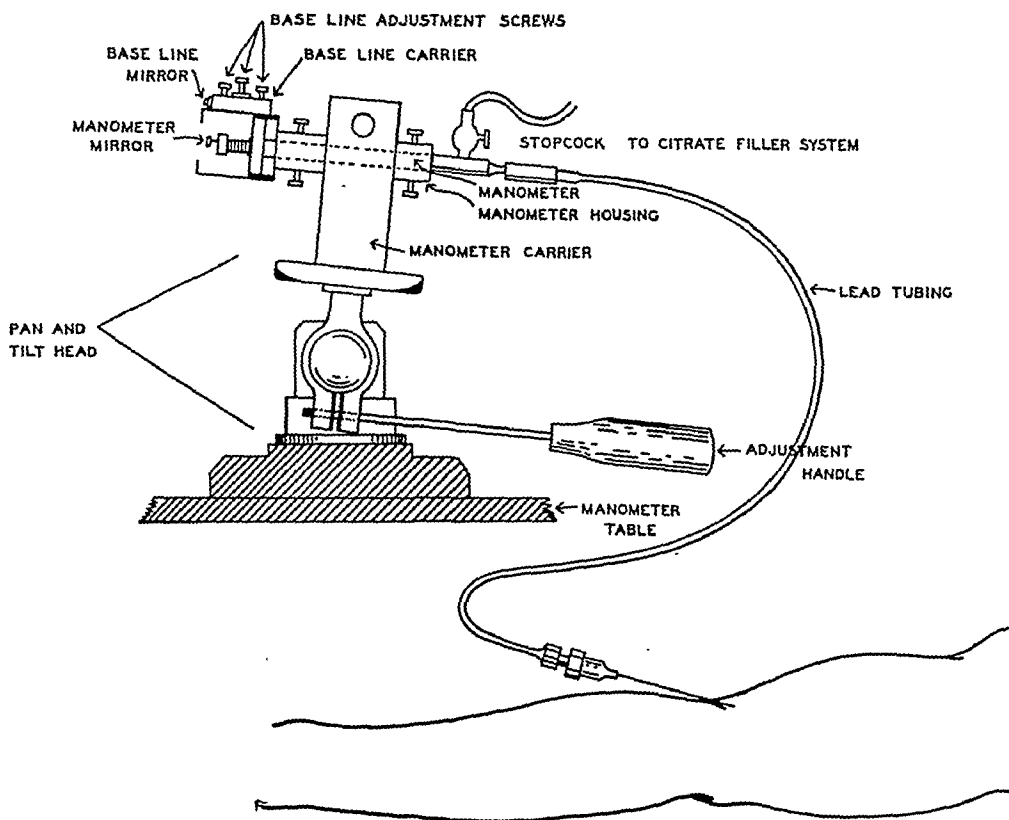


Fig. 1.—Manometer assembly (side view).

distance at two meters and the most favorable mass of a system yielding adequate natural frequencies for membranes in excess of 0.001 inch in thickness as follows: manometer,  $\frac{3}{16}$  inch in diameter,  $4\frac{3}{8}$  inches in length; lead tube, approximately 42 inches in length,  $\frac{1}{8}$  inch in diameter; 18- or 20-gauge needles. Employment of the instrument in small spaces, however, demands greater compactness, shorter optical distances, and hence less sensitivity. To counteract this, it seemed plausible to increase the diameter of the manometer. Accordingly, manometers were constructed with bores  $\frac{5}{16}$  inch in diameter. Natural frequencies remained satisfactory and sensitivity was usually increased twofold for identical membrane thicknesses. The larger caliber manometers so enhanced sensitivity, and consequently analysis of records, that we now employ

all recordings. An optical lever of one meter may be used, but where greater space permits, this lever is increased to two meters.

In order to record arterial pressures accurately, it is necessary to have manometric frequencies in excess of 100 c.p.s.<sup>1</sup> Frequencies are determined when each manometer is assembled and checked at short intervals thereafter. The system is filled with sterile citrate solution and suction is applied at the open end of the needle. Suddenly releasing the pressure throws the manometer into free vibrations of its own natural frequency which may then be photographed.

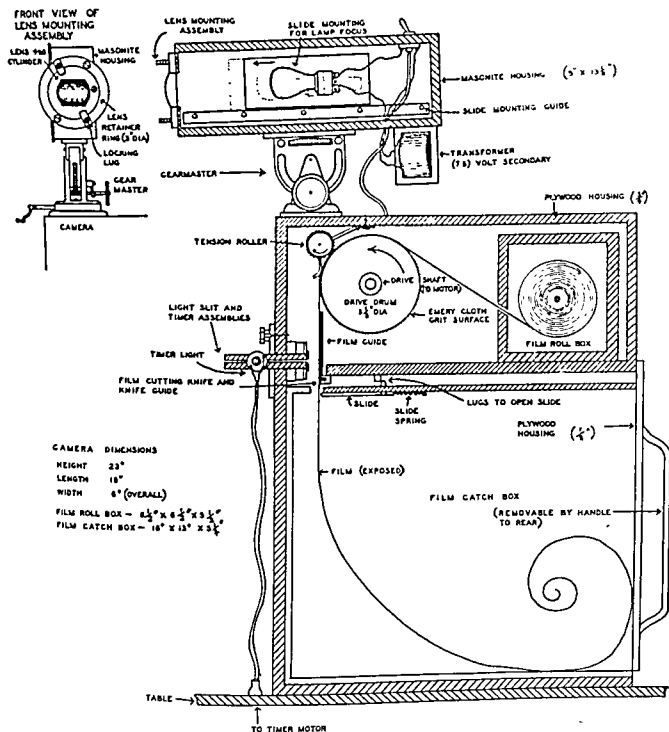


Fig. 2.—Light source and camera assemblies (left side).

**Calibration.**—Immediately upon completion of an experimental procedure, all manometers are calibrated against the mercury column. It is important that the needles remain in a position identical to that when the record was taken. They are plunged into a thick rubber tube, one end of which is clamped and the other connected to the mercury calibrating manometer, and a series of records taken as the mercury is elevated by successive increments (Fig. 4).

**Membranes.**—Many metals have been used to construct membranes. We have successfully employed both coin silver and beryllium copper in thicknesses

ranging from 0.0005 to 0.002 inch. The beryllium copper must be heat treated in a sand bath at 500° F. for one hour to assure proper "springiness." The membrane is cut to a diameter just exceeding that of the manometer. A tiny stud is eccentrically soldered to its outer surface before it is transfixed to the manometer by the cap nut. The mirrors, either front or rear surface, are cut from 0.5 D planoconvex lenses, measure approximately 4 by 4 mm., and focus sharp vertical beams of light at two meters. Ordinary rubber cement has proved satisfactory for mounting them to the membrane stud.

*Light Source.*—On top of the camera is the light box (Fig. 2) constructed of masonite  $\frac{1}{8}$  inch thick. It rests on a Bolex gearmaster allowing aiming of the beam of light in four directions. Once adjusted, locking lugs on the gearmaster prevent accidental movement of the light box. A 50 c.p. automobile headlight bulb, with its filament in the vertical position, is attached to a slide within the box which, in turn, may be moved back and forth by means of a small lever extending through a slit to the outside. Slide adjustment is necessary to focus the beam of light through the +9.0 D cylinder at the front of the light box. The cylinder, mounted in a circular retainer ring, may be rotated upon loosening two locking lugs. Its axis must be horizontal for the reflected beam to cast a horizontal rectangle of light on the manometer bank. Momentary adjustment of the bulb slide, gearmaster, and lens retainer ring is usually necessary preceding each experiment.

The masonite light box housing, together with the small bulb, prevents excessive heating of the unit and renders ventilation (and coincidental light leakage) unnecessary. Adjustment of the light beam by trial and error is simple, requiring only a few moments to obtain sharp vertical lines from the mirrors. Finally, the light source and camera have been incorporated on a single mount, facilitating compactness, mobility, and ease of alignment.

*Camera.*—The camera (Fig. 2) is housed in plywood  $\frac{3}{4}$  inch thick and is composed primarily of two parts: upper (load) and lower (film catcher) boxes. Access to the load box is through a slide panel, removable to the rear. It accommodates 175 feet of 12 cm. bromide paper which is inserted on a roll after removal of a plywood shield transfixed to the box by a wing screw. The film is brought through a slit in the front of the load box and thence over a drive drum covered with a grit-surface emery cloth. Purchase of film to the drum is aided further by a tension roller suspended (without springs) from the roof of the camera to rest upon the drive drum. A vertical strip of spring steel guides the film past the exposure slit into the film catcher box. Exposed film is cut with a knife mounted in a guide just above the film catcher box and operated by a lever attached to the side of the camera (Fig. 3). When the catcher and its exposed film is removed, further exposure is prevented by a slide and spring arrangement which closes the slit through which the film entered the box.

A diagram of the light slit in the camera may be seen in Fig. 2. The entire assembly, together with the timer light, is constructed as a single unit attached to the face of the camera by a transfixion screw above. The width of the slit just in front of the recording paper is approximately  $\frac{1}{64}$  inch. The tunnel in front of it prevents dispersion of light among the several beams and reduces the parallax.

The timer light (Fig. 3) is constructed to cast a point of light on the side of the film each second (Fig. 4, lowest record). A telechron motor beneath the camera table, operating from a 115-volt A.C. primary circuit, describes 1 r.p.s. It is equipped with a contact arm activating, through a transformer, a small

bulb at one end of the light slit. A tiny mirror reflects this light through a 27-gauge hypodermic needle to the film.

The motor, mounted on the side of the camera, is a small Holtzer-Cabot type operating from a 115-volt A.C. circuit through 60 cycles. It is so geared to the drive drum that camera speeds of 2 and 6 mm. per second are available. The faster speed is adequate for recording contour variations in arterial pressure, whereas the slower speed is more convenient for analyzing (in absolute systolic and diastolic pressures) slow changes over longer periods of time.

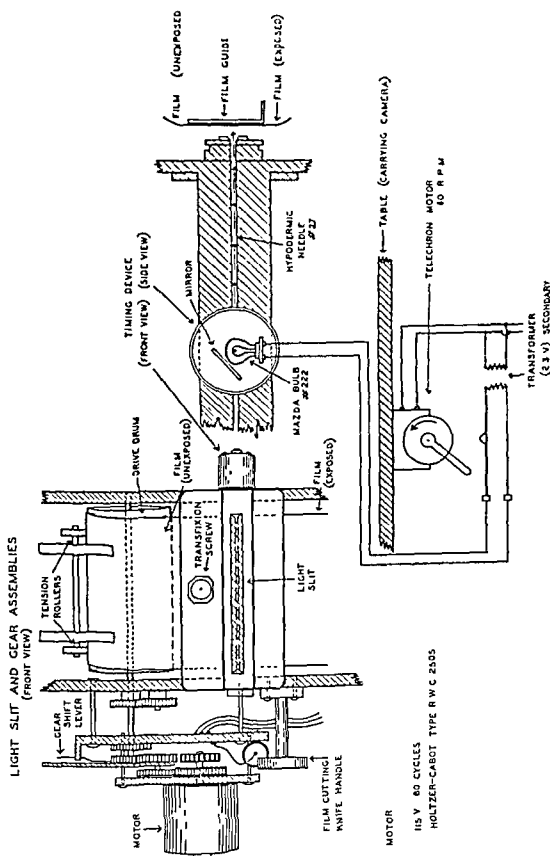


Fig. 3.—Details of gear assemblies in camera and of timing device.

*Records.*—In Fig. 4 is a simultaneous record of the intrapulmonic pressure, the arterial pressure, and the venous pressure during the performance of a Valsalva experiment in man after a deep inspiration (called "Vi" maneuver). For the intrapulmonic pressure an air-filled system was used; for the other pressures a fluid-filled system was used. Thicknesses of the membranes used depends on the sensitivity desired. In this record, taken at an optical distance of

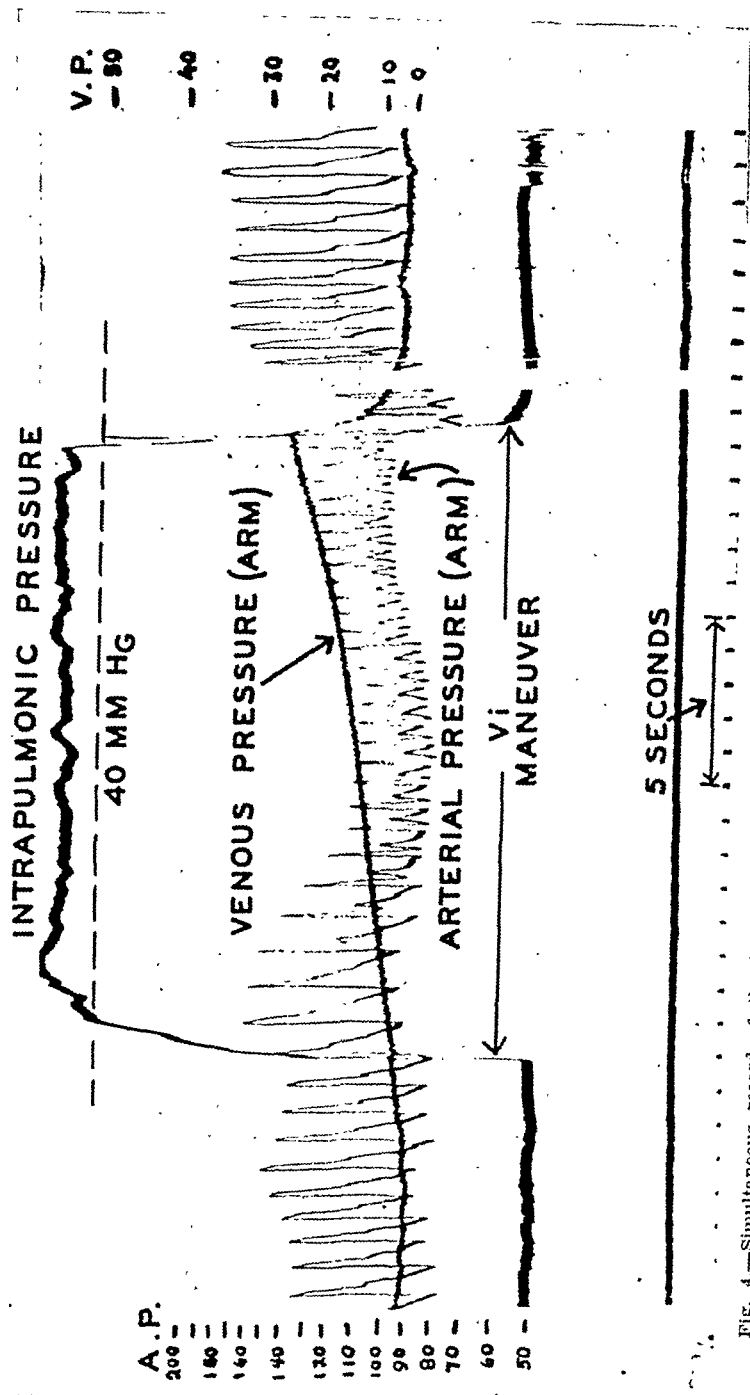


Fig. 4.—Simultaneous record of the intrapulmonic pressure, the brachial arterial pressure, and the ante-cubital venous pressure recorded with the modified optical manometer during the performance of a Valsalva maneuver by a human subject after a deep inspiration (Vi maneuver). A.P., Arterial pressure; V.P., venous pressure.

two meters, the membranes were of the following thicknesses: arterial pressure, 0.0015 inch; venous pressure, 0.00075 inch; intrapulmonic pressure, rubber dam.

### CONCLUSIONS

1. The Hamilton manometer was modified for use in limited spaces for recording arterial and venous pressures particularly.

2. Changes were made in the mounting, the bore, and the method of calibration of the manometer and in the camera and source of light.

The authors are grateful to Mr. Frank V. Garbich, who did the construction; Lt. S. Rodbard, for suggesting the use of the pan and tilt head on the manometer; Dr. W. F. Hamilton, of Augusta, Ga., and Dr. L. N. Katz, of Chicago, for suggestions and essential parts; Technician 4th Grade Walter Wagner, for technical assistance; and Sgt. Ted Wade, for the sketches.

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## A GUINEA PIG HOLDER FOR JUGULAR AND CARDIAC NEEDLING

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A BIVALVED plaster cast has been found much more convenient than the conventional animal board for jugular and cardiac needling. Animals are immobilized more quickly and more thoroughly. Much time is saved and fewer



Fig. 1.—R, Respiratory vent; S, setscrew; W, wire hook.

From the Allergy Clinic, Department of Internal Medicine, University of Virginia Medical School.  
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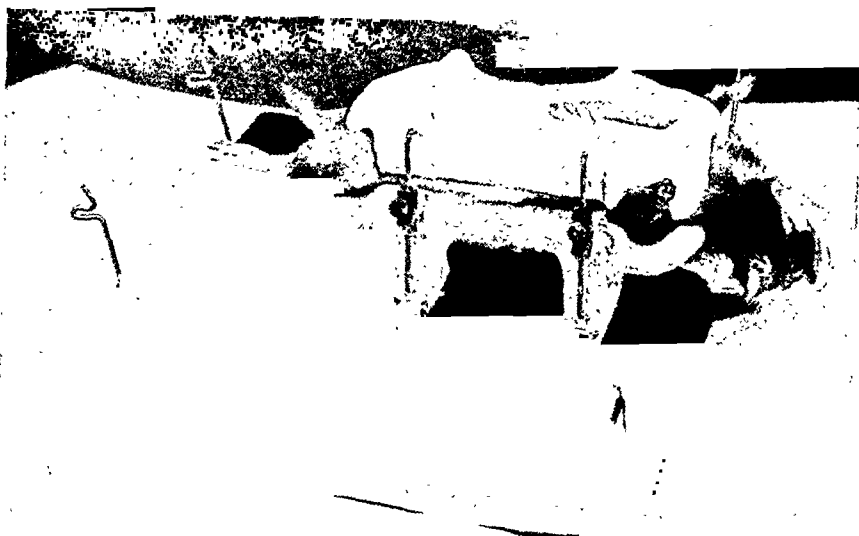


Fig. 2.

veins are torn by struggling during injections or aspirations. The cast is cut out so that it is not soiled by urine or feces.

The essential details of the holder are illustrated in Figs. 1 and 2. Variation in size of the pigs is accommodated for by the opening at the posterior end and by separation of the two halves. With small pigs a snug fit is obtained by placing a cloth pad beneath them. The wire hooks are used to immobilize the legs for femoral and saphenous punctures. The hooks are not necessary for jugular or cardiac needling.

## PRECIPITIN REACTION: QUANTITATIVE MEASUREMENT WITH A MERCURY SEALED CAPILLARY "J" TUBE

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JOHNSON and associates<sup>1</sup> described a modification of the Meloche micro-centrifuge tube<sup>2</sup> used in the quantitative measurement of precipitin reactions. They used paraffin and a broad rubber band over the ends instead of sealing the capillary tube in a globule of mercury as was done by Meloche. They also reviewed the literature on this subject.

This report describes another modification of the tube which retains all the advantages previously recognized and greatly simplifies the method of sealing and cleaning.

The capillary section of the tube is made about 5 cm. longer than usual to permit bending in the form of a "J." A small filling bulb is put in the end. A drop of mercury put in this bulb effectively seals the lower bend of the "J."

From the Allergy Clinic, Department of Internal Medicine, University of Virginia Medical School.

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The antigen-antibody mixture is placed in the large end of the tube. Adequate centrifugation packs the precipitate down on the mercury. The apparent volume of precipitate can be measured quantitatively with the tube in the vertical position (height  $\times \pi r^2$ ).

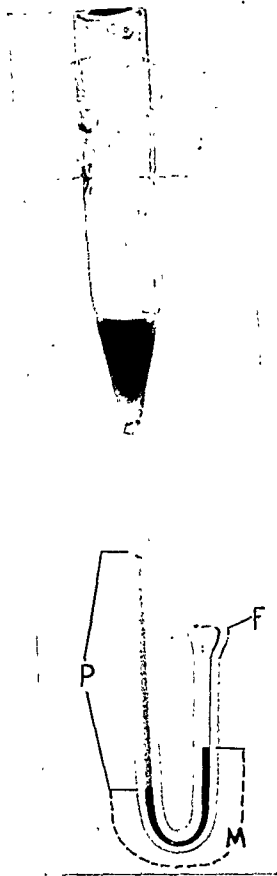


Fig. 1.—P, Precipitate; M, mercury; F, filling bulb.

To clean the tube simply empty the mercury and suck cleaning fluid through it.

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2. See reference 8 of Johnson and others.<sup>1</sup>

# A POLYVALENT SERUM FOR THE DIAGNOSIS OF SALMONELLA

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THERE is no means of avoiding the use of a large set of sera for the typing of *Salmonella*. It is, however, impossible for a small laboratory to carry such a number of sera in stock. In order to meet the requirements of smaller establishments, it is desirable to provide a serum which will give a positive reaction with most of the known types of *Salmonella*. Such polyvalent diagnostic serum containing agglutinins for the agglutinogens of *Salmonella* found in the United States has been described by Galton and Quan.<sup>1</sup> Experience of the authors with *Salmonella* strains received from the tropics and subtropical regions called their attention to the necessity of widening the range of activity of the polyvalent sera to include less common strains.

A new polyvalent diagnostic serum was devised therefore which contains antibodies for one or more of the antigenic factors of all known *Salmonella* strains, with the exception of three rare types: *Salmonella Cerro*, *Salmonella Arizona*, and *Salmonella Ballerup*. The serum is prepared as follows:

1. Motile cultures of *Salmonella*, checked with the aid of some appropriate method, for example, that of Hajna,<sup>2</sup> are inoculated separately into nine tubes containing measured amounts of 1 per cent sterile proteose-peptone broth with  $\frac{1}{2}$  per cent sodium chloride, pH 7.2 to 7.4. The *Salmonella* listed in Table I are used.

After twenty-four hours of incubation at 36 to 37° C., 0.85 per cent saline containing 0.6 per cent formalin is added to the tubes. The amount of the formol-saline should be equal to the volume of the broth in the tubes. The contents of the tubes are poured together into a sterile flask and preserved in the ice-box.

TABLE I

SALMONELLA	ANTIGENIC STRUCTURE		
<i>S. paratyphi</i> A	I, II, XII	a	
<i>S. schottmuelleri</i> (Variety java)	IV, V, XII	b	
<i>S. typhimurium</i>	IV, XII	i	1, 2, 3 . . . . .
<i>S. Hartford</i>	VI, VII	y	e, n, z <sub>16</sub> . . . .
<i>S. Muenchen</i>	VI, VIII	d, d <sub>3</sub> , d <sub>4</sub>	1, 2 . . . . .
<i>S. enteritidis</i>	I, IX, XII	g, o, m, z <sub>1</sub> , z <sub>2</sub>	
<i>S. London</i>	III, X, XXVI	l, v	1, 4, 6 . . . . .
<i>S. Selandia</i>	III, XV	e, h	1, 7 . . . . .
<i>S. Senftenberg</i>	I, III, XIX	g, s, t, z <sub>8</sub> , z <sub>9</sub>	

At least two rabbits are inoculated into the ear vein with 0.5, 1.0, 2.0, and 2.0 c.c. of this mixed "H" antigen every fourth day.

2. The surface of eight nutrient agar slants, containing 1 per cent proteose-peptone,  $\frac{1}{2}$  per cent sodium chloride, and 1.5 per cent agar, pH 7 to 7.2, are inoculated heavily with the *Salmonella* strains listed in Table I, with the exception of *Salmonella typhimurium*. After twenty-four hours of incubation at 36 to 37° C., the surface growth is washed off with small amounts of absolute ethyl alcohol and the washings from all tubes collected into one flask and mixed

From the Enteric Center of the Chicago Medical School.  
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and distributed into centrifuge tubes. The tubes are kept for one hour at 56° C. in the water bath, are centrifuged, and the alcohol drained off and substituted with equal volumes of 0.85 per cent saline containing 0.3 per cent formalin. This mixed "O" antigen may be preserved in the icebox for several weeks.

The rabbits treated with the mixed "H" antigen receive 0.5, 1.0, and 2.0 c.c. of this antigen every fourth day.

3. Finally, the rabbits are given intravenous injections of 0.5, 1.0, and 1.0 c.c. of a mixture of live, twenty-four-hour-old proteose-peptone broth cultures of all *Salmonella* strains listed in Table I. After test bleeding, the serum is harvested and preserved by the addition of an equal volume of glycerin. It is used both for slide and for tube agglutination tests, using the known and reliable technique of Edwards and Bruner.<sup>3</sup>

The serum described gave positive results with 97 stock cultures and 276 freshly isolated strains belonging to the groups "A" to "E" of *Salmonella* in dilutions 1:3 and higher, when the slide method was used. It agglutinated 110 stock cultures and 293 freshly isolated *Salmonella* strains, members of the groups "A" to "F," in dilutions 1:160 and higher, when the test tube method was applied. Two strains of *S. Ballerup* and one strain of *S. Cerro* were not agglutinated by this serum.

It is believed that this polyvalent serum may aid in the simplification of enteric work both in laboratories handling specimens from the United States and from abroad.

#### SUMMARY

The preparation of a polyvalent diagnostic serum for *Salmonella* is given. This serum may be used for slide agglutination tests of *Salmonella* belonging to the groups "A" to "E." It gives positive test tube agglutination reactions with *Salmonella* which are members of these groups and with most strains belonging to group "F," i.e., with about 99 per cent of all known *Salmonella* types.

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## THE USE OF TEST PAPER FOR THE RAPID ESTIMATION OF SULFONAMIDES IN BLOOD AND OTHER BODY FLUIDS

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SANITARY CORPS, UNITED STATES ARMY

A SIMPLE and rapid method for estimating the level of sulfonamide in blood is of much clinical value. The importance of knowing the concentration of free drug circulating in the blood stream of sulfonamide-treated patients is unquestioned. Colebrook<sup>1</sup> has pointed out that a therapeutic sulfonamide level is desirable and more effective at the onset of a bacterial infection and that the action of the drug is favored by fever temperatures.

Quick methods for estimating sulfonamides should retain the accuracy of the older methods, reduce the number of steps used, and, if possible, minimize the inconvenience to the patients. In a previous communication<sup>2</sup> I reported a rapid method using a test paper for estimating the concentration of sulfonamides in serum. The method differed radically from the usual procedures, and the preliminary results obtained indicated several points of superiority over tests in current use. The object of the present paper is to present data obtained in the actual clinical application of the method in an Army General Hospital. Results are reported on 518 samples of blood from patients being treated for a variety of diseases in which sulfonamide values were obtained by two methods. In each case the test paper value was used as a check on a parallel test run with the procedure of Bratton and Marshall.<sup>3</sup> Incidentally, by predicting the sulfonamide level of the unknown with the test paper, it was possible to set up only one standard of a value in close proximity to that of the unknown when using the Bratton and Marshall method.

In my previous communication two questions were raised: (1) whether the test paper maintains its integrity over a period of time and (2) what effect varying concentrations of proteins in serum, as well as alterations of proteins in pathologic conditions, might have on the color produced, which might lead to inaccuracies. We are now ready to report more fully on these questions. Because the method has proved of real value in this hospital, a more detailed description of the procedure will be given.

The test makes use of Ehrlich's reagent, p-dimethylamino benzaldehyde, in the formation of a Schiff base with primary aromatic amines, as first described by Kuhnan in 1938.<sup>4</sup> The test paper contains this reagent with a sufficient quantity of oxalic acid to give a pH of 2.5 when wet with serum or other biologic fluid. By the use of the test paper, the level of sulfonamide can be estimated directly in plasma, serum, and other clear biologic fluids, without the addition of precipitating or acidifying agents. The orange-yellow color produced is evaluated by comparison with an appropriate color chart or with colors produced simultaneously from solutions of known concentrations.

#### PREPARATION OF TEST PAPER

Large pieces of a good grade of white absorbent paper,\* 20 by 4½ inches, are soaked in the reagent prepared as follows: 1 Gm. of a good grade of commercial p-dimethylamino benzaldehyde (straw colored) is dissolved in 2 c.c. of concentrated hydrochloric acid and to this are added 98 c.c. of a 2.23 per cent chemically pure oxalic acid. The papers are drained of excess fluid and hung up to dry. They are now stored in the dark in covered glass jars for from five to ten days, during which time most of the hydrochloric acid evaporates. The oxalic acid remaining in the papers is sufficient to give a pH of approximately 2.5 when mixed with body fluids. Once the papers are impregnated, they should not be permitted to come in contact with the skin. The edges of the papers are trimmed off and they are then cut into strips 50 by 8 mm. and stored in suitable lightproof vials. The strips are almost colorless and they will remain without discoloration for over one year. After long standing the test strips become somewhat brittle but this does not reduce their usefulness.

#### COLOR STANDARDS

A useful adjunct to the test paper is a color chart with standardized color strips. The colors are best reproduced with water colors adjusted to the proper

\*A satisfactory paper is Reeve Angel Filter Paper No. 201, 20 by 20 inches, distributed by Reeve-Angel & Co., New York, N. Y.

color by comparison with test strips moistened under the usual conditions with serum containing known amounts of sulfonamide. The standard color strips are mounted on a cardboard of purple hue in order to obtain good contrast. In the absence of a color chart the color comparison may be made with strips wet simultaneously with standard solutions. Standard solutions of sulfonamide in serum keep indefinitely when preserved with merthiolate or metaphen. A single color standard is suitable for the estimation of sulfapyridine, sulfathiazole, and sulfadiazine; for sulfanilamide the values read from this standard should be divided by 1.5. The values for other sulfonamides can be calculated similarly on the basis of molecular equivalence. The color standards are selected in intensities corresponding to 0, 3, 6, 9, 12, 15, and 20 mg. of sulfonamide per 100 c.c. or in other convenient groupings, intermediate values being estimated by interpolation.\*

#### PROCEDURE

Estimation of the level of sulfonamide is made by spreading the solution to be tested evenly over one end of the test strip by means of a fine dropper. Capillary pipettes drawn out from glass tubing are suitable. In this way, the fluid is spread over the paper more by movement of the dropper than by capillarity of the paper fibers, and reagents are not leached out of areas which are oversaturated with the test fluid. Within ten seconds a maximum development of color occurs and the test is evaluated within thirty seconds. To avoid translucence of the paper interfering with the reading, the wet strip is placed on white paper or a porcelain plate. The color is then compared with the graded series of color standards to obtain the sulfonamide value. The color ranges from a pale violet or gray with normal human plasma or serum to a bright orange-yellow with material containing from 15 to 20 mg. sulfonamide per 100 c.c. By a fortunate circumstance, the usual concentrations of sulfonamides attained in the body fall within the range of easily evaluated color intensities. For accurate reading of the colors the operator should be seated comfortably and he should receive evenly diffused light, such as the north light. Facility with the method is attained by practicing with sera of known concentrations for several sessions. Technicians usually become expert in making accurate readings within three or four days. In any event, in the hands of the clinician experienced in the interpretation of sulfonamide levels, plasma or serum concentrations should be readily classifiable into groupings such as "zero," "subtherapeutic," "therapeutic," and "toxic."

Proteins and other substances usually found in normal or pathologic plasma do not interfere with the test, nor do varying concentrations of proteins over a wide range affect the color. In the case of oxalated blood, the test is run on plasma, which is readily obtained by permitting the red cells to settle out on standing or by centrifuging. Serum can also be used and gives the same value. Sulfonamide values for whole blood are not transferable to plasma or serum values, since the latter will be a little higher than the whole blood value. The value given to a standard serum is obtained by a sulfonamide determination run on the corresponding whole blood by the Bratton and Marshall method.

In Table I is shown a comparison of values obtained by the test paper with those secured by the method of Bratton and Marshall. It may be noted that there are very few discrepancies in the values; the maximum deviations are in the higher concentrations and these do not exceed 3 mg., with the great majority

\*With the cooperation of the LaMotte Chemical Products Co., Towson, Baltimore, Md., a test kit for this test has been prepared containing color chart, test paper, and capillary tubes.

of the tests within 1 mg. or better. Although the method is subject to the usual errors of visual colorimetry, readings which agree exactly or come within 1 mg. of the Bratton and Marshall method are obtained after some practice. This small discrepancy does not interfere with the accuracy necessary for clinical appraisal of drug therapy, and no false positive values have been encountered.

TABLE I

NUMBER OF BLOOD SAMPLES	BRATTON AND MARSHALL METHOD (MG. %)	LA ROSA TEST PAPER METHOD SHOWING NUMBER OF TESTS DEVIATING BY:			
		<1 MG. %	1 MG. % ±	2 MG. % ±	3 MG. % ±
Sulfadiazine					
	Range				
5	0	5	0	0	0
22	<1	22	0	0	0
56	1-3	51	3	2	0
203	4-8	163	31	9	0
187	9-16	129	43	12	3
14	17-20	5	5	3	1
Sulfathiazole					
2	0	2	0	0	0
4	<1	4	0	0	0
2	1-3	2	0	0	0
20	4-8	13	5	2	0
3	9-13	1	1	1	0
Total 518		397	88	29	4
100%		76.6%	17.0%	5.6%	0.8%

The use of test paper for the sulfonamide determination of blood has a number of advantages over other methods in use at present. Because the volume of plasma required for the test is small, it is not necessary to effect a venipuncture to obtain a sample of blood. Blood drawn from a finger puncture is sufficient for the test. The volume of blood necessary is no more than 0.01 c.c. and this is conveniently collected in a capillary tube 68 mm. long with inside diameter not less than 1 mm.,\* containing dried oxalate to prevent coagulation. Nonoxalated capillary tubes may also be used for collecting samples of blood, but sufficient time must be allowed for complete coagulation before proceeding with the test. The oxalated tubes are prepared by filling the capillary tubes with 3 per cent potassium oxalate, followed by drying in a horizontal position in an oven at about 70° C. A large number of these tubes is prepared at one time and, if the diameter is of proper size, no clogging of the tubes is experienced. The capillaries are filled two-thirds full by contact with the drop of blood, and the clear end is then sealed by flaming. The plasma is separated in a centrifuge and the tube is cut at the junction between cells and plasma. The plasma is then transferred to the test paper by holding the open end of the tube perpendicular to the test paper and guiding it down the strip. Transfer of the fluid is easily effected by gravity and the capillary action of the paper. For identification the capillaries are flagged with gummed labels bearing numbers or names. The practicability of this procedure has been demonstrated in our laboratory in a large number of cases. It is especially convenient for use with individuals whose veins are difficult to reach, as well as with babies being treated with sulfonamide. In the case of children, the heel is usually the site chosen for puncture. The method is rapid and reliable as well as economical of equipment and man power, thus making it possible to run several tests on the same in-

\*Capillary tubes of these dimensions are supplied by the Kimble Glass Co., Vineland, N. J.

dividual during the course of the day without overtaxing the laboratory personnel. Moreover, a finger puncture is less objectionable and the patient is less disturbed.

The test paper can be used in like manner for the estimation of sulfonamides in other clear body fluids, such as spinal fluid, chest fluid, synovial fluid, and urine. Variation in the amount of protein in the test fluid does not affect the intensity of the colors produced. During chemotherapy the concentration of the sulfonamides in urine is normally too high for a direct quantitative reading, and it is necessary to dilute the urine 1 to 20 to bring the concentration within the range of color evaluation. By confining the test to undiluted urine, important information may be obtained concerning the presence or absence of sulfonamide drug in the body, as in the case of patients who have been transferred recently, or in those receiving prophylactic doses for the prevention of meningitis, scarlet fever, venereal disease, etc.

The reaction on the test paper is given by free sulfonamides and other primary aromatic amines including *p*-aminobenzoic acid; that is, by the same compounds that give color in the method of Bratton and Marshall. Urea in large concentrations such as are found in urine gives a delayed reaction which is easily distinguishable from a true sulfonamide color reaction. Sulfonamide crystals found in urine are in the conjugated form and when brought into solution do not give a positive test. Pyrrols and indoles do not react with the reagent at the acidity maintained in the test. Moderate hemolysis or pigmentation of body fluids seems not to interfere seriously with color matching. Changes in composition of plasma due to pathologic conditions are not reflected in the color produced and lead to no inaccuracies. Thus the test paper appears to be of general use for the detection of sulfonamides.

#### SUMMARY

A method is presented for the estimation of the sulfonamide level in blood and other body fluids by the use of test papers impregnated with *p*-dimethylamino benzaldehyde. The validity of this method has been demonstrated on 518 blood samples by comparing the test paper values with those obtained by the Bratton and Marshall method. The color developed on the test paper is evaluated by comparison with an appropriate color chart or colors developed with sera of known sulfonamide concentration. The method can be used with small volumes of blood drawn in capillary tubes from finger punctures. The test is applicable to plasma, serum, and other clear biologic fluids.

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## BOOK REVIEWS

**Patients Have Families.** By *Henry B. Richardson*, M.D., F.A.C.P., Associate Professor of Clinical Medicine, Cornell University Medical College; Attending Physician, New York Hospital; Visiting Physician, Bellevue Hospital, New York, N. Y. The Commonwealth Fund, 1945, New York, N. Y. Price \$3.00. Cloth with 408 pages.

This book is a report of a research effort ("The Family Study") supported by the Josiah Macy, Jr. Foundation; the cooperating organizations are Cornell University Medical College-New York Hospital (medicine, psychiatry, public health, medical social service) and Community Service Society (family service, educational nursing). An anthropologist took part during the last months of the two-year study.

During the two years of conferences among the members of the cooperating professions, there developed "a capacity to communicate with each other, to frame questions and to attempt answers," of which they had hardly been aware. It was decided therefore that the report of this work be addressed primarily to the medical profession and to the lay world concerned with medical problems and that it present (a) the value to the medical profession of seeing the individual patient as a personality and as part of his family constellation and (b) the essential contribution which can be made by psychiatry and the social disciplines to this view of the patient and to treatment plans geared to such an emphasis.

The book consists of the experiences which led to the group convictions, an attempt at setting up concepts in terms of which types of family units and family equilibria can be described and uniformities (if such exist) sought for and descriptions of the functions, procedures, and attitudes of the various co-operating professions.

Only about fifteen families were considered at all systematically by the entire group, and these for diverse reasons. Hence it is explicitly stated that the families described are in no way representative of any given area or cultural group.

One of the striking features of the report is the careful description of the history of one family known to various of the professions (chiefly in the hospital). It required two years of intensive medical studies of several of the family members before there appeared in the medical report the slightest hint that their illnesses had any relation to factors not limited to the single organism. The hint was "Inadequate intake of food due to poverty." It required four years before there appeared in the record of one family member a definite statement of a relation between that member's symptoms and "emotional stress." Thereafter, single interviews by psychiatrists and social workers brought out the fact that the supposed "medical" symptoms were temporarily related to difficulties in the interpersonal relations of the family members within the family and outside of it. Treatment could then proceed. Within a statable short period of time the problems of a number of persons could be brought to such a point that the endless waste of medical, social, community and family time, resources, and money



was stopped. Psychiatrists who act as consultants to hospital medical services make such observations regularly, but no one has collected the data systematically and expressed them in terms of such objective criteria as expenditure of time, etc. Until this is done the inefficiency of present-day medicine as compared with its potential effectiveness under the same conditions has not been demonstrated convincingly.

The description of the same family brings out another striking feature of present-day medicine, not stressed by the author of the report. Cornell University Medical College-New York Hospital has had within its walls for some ten years a research group making fundamental contributions to the viewpoint stressed by the author of the report—that of “psychosomatic” medicine. Since 1932 there has also been available a psychiatrist assigned full time by the Payne Whitney Clinic for work as consultant on the medical pavilions. Nevertheless, (about 1940) four years were required by the medical in- and outpatient departments to reach conclusions that demonstrably could be reached in perhaps four interviews, at the most, of one hour each, and were in fact so reached. The inference is warranted that even a first-rate research and teaching arrangement may accomplish little toward the effective teaching of the attitude that the patient must be considered as a behavioral unit and as a member of an interdependent system of such units. Obviously more needs to be known about the elements essential to success. A factual description of the physical setup, organizational relations, and interpersonal relations characterizing the medicine-psychiatry-social work arrangement at any one place such as Cornell Medical College-New York Hospital might be most illuminating.

It is difficult to detect any trace of anthropological influence in that section of the report dealing with the group's attempts to characterize “family equilibrium.” The usefulness of the concepts employed is likely to be extremely limited, for they are in no way defined in terms of the operations necessary to establish them. Examples of the concepts are imitation, identification, reciprocating system, focus. Attempts to define in terms of operations the equilibria of pairs of interacting individuals, of families, and of societies have already been made by anthropologists; there is no reference to any of these attempts, some of which have considerable promise.

The book has much of interest in it. It represents a first approach to the scientific study of a most important subject.

GEORGE SASLOW.

**Approved Laboratory Technic. Clinical Pathological, Bacteriological, Mycological, Virological, Parasitological, Serological, Biochemical and Histological.** By *John A. Kolmer, M.S., M.D., Dr. P. H., ScD., LL.D., L.H.D., F.A.C.P.*, Professor of Medicine in the School of Medicine and the School of Dentistry, Temple University; Director of the Research Institute of Cutaneous Medicine; Formerly Professor of Pathology and Bacteriology, Graduate School of Medicine, University of Pennsylvania; and *Fred Boerner, V.M.D.*, Associate Professor of Clinical Bacteriology, Graduate School of Medicine and Assistant Professor of Bacteriology, School of Medicine, University of Pennsylvania; Bacteriologist, Graduate Hospital, Philadelphia. Fourth Edition. D. Appleton-Century Company, Inc., New York, N. Y. Price \$10.00. Cloth with 1017 pages.

This volume is a veritable encyclopaedia of the most useful clinical laboratory procedures and their normal values. It is intended as a companion volume

to Dr. Kolmer's *Clinical Diagnosis by Laboratory Examinations* and contains; therefore, very little discussion of the significance and interpretation of abnormal results. New sections have been added in this fourth edition on methods for examining saliva, pancreatic function, blood and urine for hormones and vitamins, and stools, blood, and tissues for parasites. Other chapters have been rewritten to include new techniques like the very useful copper sulfate method of Phillips and his co-workers for determining the specific gravity of whole blood and plasma. The material on bacteriologic, mycologic, virologic, and serologic methods is particularly well done. There are valuable unique sections like the ones on the care, inoculation, and bleeding of laboratory animals, recognition of the common diseases of laboratory animals, and the prevention and treatment of laboratory accidents. The book is unusually well printed for a war volume, is profusely illustrated, and contains a wealth of charts. The style is simple, direct, and easy to read.

Hospital laboratories, clinical pathologists, and medical technologists will find the book an essential reference work. Since it must be used in conjunction with the above-mentioned companion volume for clinical purposes, however, the expense involved makes it less serviceable as a textbook for medical students.

**The Marihuana Problem in the City of New York.** By the *Mayor's Committee on Marihuana*. The Jaques Cattell Press, Lancaster, Pa. Price \$2.50. Cloth with 220 pages.

